THE
BIOLOGICAL BULLETIN

PUBLISHED BY
THE MARINE BIOLOGICAL LABORATORY

Editorial Board

T. H. BULLOCK, University of California, Los Angeles
E. G. BUTLER, Princeton University
K. W. COOPER, University of Rochester
L. V. HEILBRUNN, University of Pennsylvania
M. E. KRAHL, University of Chicago
J. H. LOCHHEAD, University of Vermont
E. T. MOUL, Rutgers University
ARTHUR W. POLLISTER, Columbia University
MARY E. RAWLES, Johns Hopkins University
BERTA SCHARRER, Albert Einstein College of Medicine
J. H. WELSH, Harvard University
A. R. WHITING, University of Pennsylvania

DONALD P. COSTELLO, University of North Carolina
Managing Editor

VOLUME 110
FEBRUARY TO JUNE, 1956

Printed and Issued by
LANCASTER PRESS, Inc.
PRINCE & LEMON STS.
LANCASTER, PA.

THE BIOLOGICAL BULLETIN is issued six times a year at the Lancaster Press, Inc., Prince and Lemon Streets, Lancaster, Pennsylvania.

Subscriptions and similar matter should be addressed to The Biological Bulletin, Marine Biological Laboratory, Woods Hole, Massachusetts. Agent for Great Britain: Wheldon and Wesley, Limited, 2, 3 and 4 Arthur Street, New Oxford Street, London, W. C. 2. Single numbers $2.50. Subscription per volume (three issues), $6.00.

Communications relative to manuscripts should be sent to the Managing Editor, Marine Biological Laboratory, Woods Hole, Massachusetts, between June 1 and September 1, and to Dr. Donald P. Costello, Department of Zoology, University of North Carolina, Chapel Hill, North Carolina, during the remainder of the year.

Sci
QH
301
B59
vol 110-111
1956

Entered as second-class matter May 17, 1930, at the post office at Lancaster, Pa., under the Act of August 24, 1912.

LANCASTER PRESS, INC., LANCASTER, PA.

91c

CONTENTS

No. 1. February, 1956 PAGE

Berg, William E.
Cytochrome oxidase in anterior and posterior blastomeres of Ciona intestinalis 1

Burden, Charles E.
The failure of hypophysectomized Fundulus heteroclitus to survive in fresh water 8

Dan, Katsuma, and Kayo Okazaki
Cyto-embryological studies of sea urchins. III. Role of the secondary mesenchyme cells in the formation of the primitive gut in sea urchin larvae 29

Dehnel, Paul A.
Growth rates in latitudinally and vertically separated populations of Mytilus californianus 43

Johnson, Martin W., and Robert J. Menzies
The migratory habits of the marine gribble Limnoria tripunctata Menzies in San Diego harbor, California 54

Krugelis–Macrae, Edith
The occurrence of porphyrin in the planarian 69

Ludwig, Daniel, and Mary C. Barsa
Oxygen consumption of whole insects and insect homogenates 77

Nutting, W. L.
Reciprocal protozoan transfaunations between the roach, Cryptocercus, and the termite, Zootermopsis 83

Sussman, Maurice
On the relation between growth and morphogenesis in the slime mold Dictyostelium discoideum 91

Yost, Henry T., Jr., Hope H. Robson and Irwin M. Spiegleman
Studies on the effects of irradiation of cellular particulates. I. Inhibition of cytochrome oxidase by ultraviolet 96

No. 2. April, 1956

Costlow, John D., Jr., and C. G. Bookhout
Molting and shell growth in Balanus amphitrite niveus 107

Davis, H. C., and P. E. Chanley
Spawning and egg production of oysters and clams 117

Engels, William L., and Charles E. Jenner
The effect of temperature on testicular recrudescence in Juncos at different photoperiods 129

FINGERMAN, MILTON, AND CAROL FITZPATRICK
An endocrine basis for the sexual difference in melanin dispersion in Uca pugilator 138
FINGERMAN, MILTON, AND DONALD W. TINKLE
Responses of the white chromatophores of two species of prawns (Palaemonetes) to light and temperature 144
HEILBRUNN, L. V., AND WALTER L. WILSON
Antimitotic substances from the ovaries of vertebrates 153
HULBURT, E. M.
The phytoplankton of Great Pond, Massachusetts 157
JONES, RAYMOND F.
On the chemical composition of the brown alga Himanthalia elongata (L.) S. F. Gray 169
SAMUELS, ARTHUR
The effect of sex and allatectomy on the oxygen consumption of the thoracic musculature of the insect, Leucophaea maderae 179
TYLER, ALBERT, ALBERTO MONROY AND CHARLES B. METZ
Fertilization of fertilized sea urchin eggs 184
TYLER, ALBERT, AND MELVIN SPIEGEL
Elevation and retraction of the fertilization membrane of echinoderm eggs fertilized in papain solutions 196
WILLIAMS, CARROLL M.
Physiology of insect diapause. X. An endocrine mechanism for the influence of temperature on the diapausing pupa of the cecropia silkworm 201

NO. 3. JUNE, 1956

BECK, STANLEY D.
A bimodal response to dietary sugars by an insect 219
BRYAN, JOHN H. D., AND JOHN W. GOWEN
A histological and cytophotometric study of the effects of x-rays on the mouse testis 229
COLWIN, LAURA HUNTER, AND ARTHUR L. COLWIN
The acrosome filament and sperm entry in Thyone briareus (Holothuria) and Asterias 243
DAUGHERTY, JACK W.
Polyphenol oxidase activity and pigmentation in snail tissues 258
DAVISON, JOHN
An analysis of cell growth and metabolism in the crayfish (Procambarus alleni) 264
FINGERMAN, MILTON
Phase difference in the tidal rhythms of color change of two species of fiddler crab 274
FRINGS, HUBERT, AND MABLE FRINGS
The loci of contact chemoreceptors involved in feeding reactions in certain Lepidoptera 291
JØRGENSEN, C. BARKER, AND PER ROSENKILDE
On regulation of concentration and content of chloride in goldfish 300

JØRGENSEN, C. BARKER, AND PER ROSENKILDE
Relative effectiveness of dehydration and neurohypophysial extracts in enhancing water absorption in toads and frogs 306
KINGSBURY, JOHN M.
On pigment changes and growth in the blue-green alga, Plectonema nostocorum Bornet ex Gomont 310
OKAZAKI, KAYO
Skeleton formation of sea urchin larvae. I. Effect of Ca concentration of the medium 320
ROBERTS, HENRY S., AND NANCY S. JOHNSON
Cytokinesis of multi-spindle cells 334
ROGERS, K. T.
Re-examination of the production of cyclopia in Fundulus heteroclitus with magnesium chloride and ethyl alcohol 344
SIEGEL, R. W.
Mating types in Oxytricha and the significance of mating type systems in ciliates 352

Volume 110 Number 1

UNIVERSITY
OF MICHIGAN
FEB 23 1956
SCIENCE
LIBRARY

THE BIOLOGICAL BULLETIN

PUBLISHED BY
THE MARINE BIOLOGICAL LABORATORY

Editorial Board

T. H. BULLOCK, University of California, Los Angeles
E. G. BUTLER, Princeton University
K. W. COOPER, University of Rochester
L. V. HEILBRUNN, University of Pennsylvania
M. E. KRAHL, University of Chicago
J. H. LOCHHEAD, University of Vermont
E. T. MOUL, Rutgers University
ARTHUR L. POLLISTER, Columbia University
MARY E. RAWLES, Johns Hopkins University
BERTA SCHARRER, Albert Einstein College of Medicine
J. H. WELSH, Harvard University
A. R. WHITING, University of Pennsylvania

DONALD P. COSTELLO, University of North Carolina
Managing Editor

FEBRUARY, 1956

Printed and Issued by
LANCASTER PRESS, Inc.
PRINCE & LEMON STS.
LANCASTER, PA.

THOMAS Selection of Apparatus . . .

The 22,000 items listed in our Catalogue and Supplement and carried in our stock have been carefully selected upon the basis of fifty-five years of experience in providing for the needs of laboratories of Chemistry and Biology, *both large and small.*

All subjected to careful examination

All of the items have been subjected to careful examination and testing by our Technological staff. The choice is therefore based on what we can with assurance recommend as being satisfactory for the purpose intended.

Detailed descriptions

Descriptions are accurate and detailed and include accessory and replacement parts.

A.H.T. Co. Specification items

Many important specialties and groups of items such as lamp-blown and volumetric glassware, rubber tubing, HR-108 rubber stoppers, etc., generally listed under the designation "A.H.T. Co. Specification," are in accordance with our designs or specifications. Such items are in our judgment, *superior* in design, performance, accuracy, appearance or durability, to similar trade articles.

Trade marked or branded items

We list and stock many articles under the manufacturer's name or brand and, while the maker is primarily responsible for the design and performance of such apparatus, *we take full responsibility*—having exercised discrimination in our selection—for all trade marked or branded items listed by us.

Miscellaneous items

Many of the remaining items such as spatulas, brushes, corks, files, aprons, dropping pipettes, etc., are offered under neither our name nor that of the maker. They are obtainable in a variety of grades and from competing manufacturers. Many of them are not made primarily for laboratory use. The grades we have selected are of *dependable quality and uniformity* and will be found highly satisfactory for laboratory use.

More and more laboratories RELY ON THOMAS

ARTHUR H. THOMAS COMPANY

Laboratory Apparatus and Reagents

WEST WASHINGTON SQUARE
PHILADELPHIA 5, PA.

Teletype Services: Western Union WUX and Bell System PH-72

Vol. 110, No. 1 February, 1956

THE
BIOLOGICAL BULLETIN

PUBLISHED BY THE MARINE BIOLOGICAL LABORATORY

CYTOCHROME OXIDASE IN ANTERIOR AND POSTERIOR BLASTOMERES OF CIONA INTESTINALIS

WILLIAM E. BERG

Department of Zoology, University of California, Berkeley 4, California

The cytochemical test for indophenol blue oxidase, the indophenol or "nadi" reaction, has long been used to demonstrate supposed gradients or localizations of this enzyme in a wide variety of embryonic material. A particularly striking localization of this enzyme, presumably cytochrome oxidase, has been claimed for the ascidian egg where the myoplasm, presumptive for the larval musculature, gives an intense reaction with the indophenol reagents (Ries, 1937, 1939; Reverberi and Pitotti, 1939).

Cytochemical tests for demonstrating localization of enzymes in living cells are open to serious criticisms. Differential rates of staining of cells or of areas of a cell could be due to a variety of factors other than differences in enzyme activity, and it is extremely difficult to evaluate the sources of error. The aim of the present investigation was to make quantitative measurements of cytochrome oxidase in the ascidian blastomeres which stained differentially with the indophenol reagents. At the four-cell stage, the ascidian embryo consists of anterior and posterior blastomeres, the latter giving a strong oxidase reaction. Techniques were developed for separating the two types of blastomeres in sufficient numbers for quantitative analyses of cytochrome oxidase by a microspectrophotometric method. The results reported below verify the conclusions reached by previous investigators using cytochemical methods.

Methods

Gametes were obtained from *Ciona intestinalis* by removing the test and opening the oviduct and vas deferens. Chorions of the unfertilized eggs were digested off with a protease solution (3% crude protease [1] in sea water) and the "naked" eggs were washed, transferred to agar-coated dishes and fertilized. An agar coat on all glassware is necessary to prevent sticking and subsequent cytolysis of the eggs. Several hundred first-cleavage blastomeres were separated from one another and in turn segregated by means of a breaking pipette from the remaining mass of eggs. Division of each isolated first-cleavage blastomere resulted in an anterior and posterior cell which were separated with the tip of a breaking pipette and transferred to separate dishes. Although they are identical in volume the an-

[1] Purchased from Worthington Biochemical Sales Co., Freehold, New Jersey.

terior and posterior blastomeres can be distinguished by the elongated shape and clear cytoplasmic "cap" of the posterior blastomere (Castle, 1896). These characteristics are temporary and identification and separation of the blastomeres must be performed rapidly.

Cytochrome oxidase was measured by a microspectrophotometric method originally described by Cooperstein and Lazarow (1950) and repetitiously by Hess and Pope (1953). Basically, the method consists of the spectrophotometric observation of the rate of oxidation of reduced cytochrome *c* with the Beckman spectrophotometer adapted for the use of microcuvettes (Lowry and Bessey, 1946).

In more detail, the method as used for determination of cytochrome oxidase in eggs is as follows. The eggs or blastomeres were transferred by means of a breaking pipette to a 0.1-ml. conical-shaped centrifuge tube. By light centrifugation the cells were forced to the end of the tube and excess sea water was then drawn off with a pipette. Four to five μl. of homogenization medium were added, and the eggs homogenized by drawing them in and out of a fine pipette having a bore less than the diameter of an egg. The homogenization medium consisted of 0.75–1.0% sodium desoxycholate in phosphate buffer. This has been shown to have a clarifying action on crude homogenates and apparently causes a dispersal of the cytochrome oxidase complex (Hess and Pope, 1953). An attempt was made to reduce deterioration of cytochrome oxidase after homogenization by including serum protein in the homogenization medium (Borei, 1950). Although with some types of material the added protein seemed to offer some protection, its inclusion was not essential in the present experiments. Loss of enzyme activity was reduced considerably by rapid homogenization of the eggs at 3 to 4° C. Temperature control was obtained by placing the homogenization tube in a previously chilled copper block with drilled holes for microscopic observation.

The above procedure required only 20 to 30 seconds and the homogenate was then added to 45 μl. of reduced cytochrome *c* solution (1.6×10^{-5} *M* cytochrome *c*, reduced with sodium hydrosulfite, in 0.04 *M* phosphate buffer, pH 7.2) contained in a 50-μl. microcuvette. The reactants were stirred with a fine glass rod and the change in optical density at 550 millimicrons was followed for 2.5 minutes in a Beckman spectrophotometer. At the end of this period a small amount of potassium ferricyanide was added and the optical density of completely oxidized cytochrome *c* was measured. The measurements were carried out at room temperature (22 to 24° C.).

With the concentration of reactants used the reaction was first order and cytochrome oxidase activity could be expressed as the velocity constant of the reaction. The optical density of oxidized cytochrome *c* was subtracted from the readings at the various time intervals and the logarithms of these differences were plotted against time. This gave a straight line from which a velocity constant $\frac{(\Delta \log \text{ferrocytochrome } c)}{\Delta T}$ could be calculated (Cooperstein and Lazarow, 1950). Blanks (cytochrome *c* + homogenization medium) were carried out with each experiment in order that a correction could be made for the slow autoxidation of reduced cytochrome *c*.

In the present experiments the interest is in relative enzyme activities; accordingly all results are expressed as velocity constants. As can be seen from the cali-

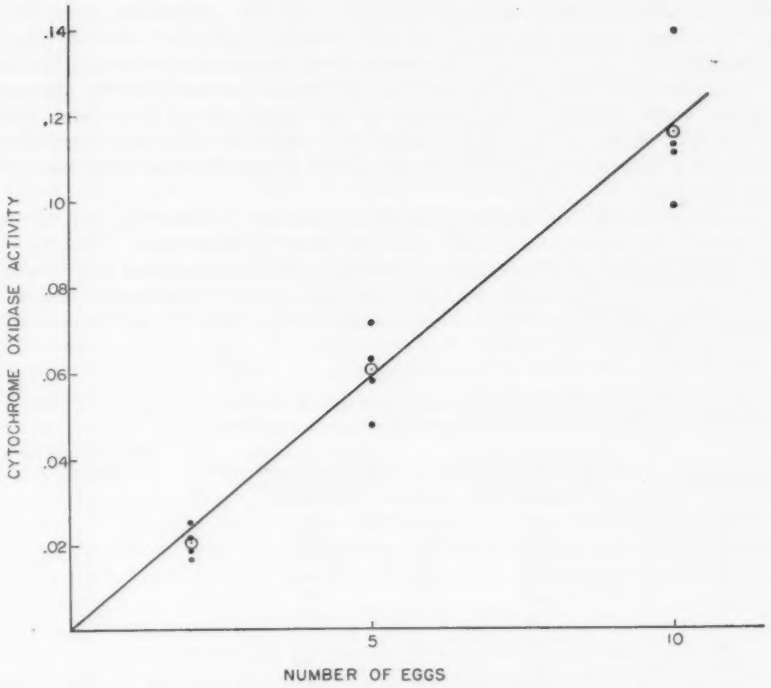

FIGURE 1. Cytochrome oxidase activity (Δ log ferrocytochrome *c*/min.) in homogenates of *Ciona* eggs. Solid points are individual measurements, large circles are averages.

bration curve (Fig. 1) these are proportional to the amount of cytochrome oxidase. The method is extremely sensitive, and it is possible to measure the cytochrome oxidase activity in a single *Ciona* egg.

RESULTS

Anterior and posterior blastomeres of *Ciona* were separated, and the cytochrome oxidase activities measured with the above described method. From previous work in determining the calibration curve it was decided that a sample containing about 20 second-cleavage blastomeres provided sufficient cytoplasm for reasonably accurate measurement of enzyme activity. In the first experiments 15 to 20 each of the anterior and posterior blastomeres were used, and the results were satisfactory. Straight lines representing reaction rates were obtained, and there was little subjective error in determining the slopes of the lines. As more skill in separating blastomeres was developed, the number used for each test was increased.

Several precautions were necessary in measuring the cytochrome oxidase of the blastomeres. A freshly prepared reduced cytochrome *c* solution tends to give high values for a standard cytochrome oxidase preparation (in these experiments, a

standard number of whole, unfertilized eggs). As the preparation ages, over a period of several hours, the values for the standard decrease. For comparative measurements of enzyme activity this error can be eliminated by running the samples simultaneously using several cuvettes, or it can be corrected by alternately measuring single samples. The average of the values should then eliminate the error due to a change of the stock cytochrome *c* solution. The latter method was chosen as it is more convenient and permits better standardization of the homogenization procedure.

In all experiments the freshly prepared cytochrome *c* was tested several times with whole eggs before it was used with the isolated blastomeres. The time between determinations of the enzyme in the two types of blastomeres was shortened by carrying the experimental procedure for both types of blastomeres up to the point of homogenization. The results, tabulated in Table I, are expressed as

TABLE I

Cytochrome oxidase activity in homogenates of anterior and posterior blastomeres of Ciona

Number of blastomeres	Cytochrome oxidase (10^3 Δ log ferrocytochrome *c*/min.) in anterior blastomeres	Cytochrome oxidase (10^3 Δ log ferrocytochrome *c*/min.) in posterior blastomeres	Ratio cytochrome oxidase activities $\left(\frac{\text{post.}}{\text{ant.}}\right)$
20	8.0	14.8	1.9
15	2.0	4.2	2.1
24	5.4	15.3	2.8
24	4.7	11.3	2.4
27	2.4	10.7	4.5
32	4.1	14.5	3.5
36	5.9	10.9	1.9
32	5.0	15.3	3.1
32	7.8	14.0	1.8
			average 2.7

velocity constants which are proportional to cytochrome oxidase activity (Fig. 1).

Since the majority of experiments were carried out under varied conditions (different batches of eggs, cytochrome *c* solutions, temperatures, etc.) it is not possible to compare velocity constants of separate experiments with any degree of reliability. The data in Table I must therefore be considered as a paired series with the results best expressed as ratios (shown in the last column of Table I). The cytochrome oxidase activity in posterior blastomeres averages 2.7 times that in anterior blastomeres, a difference which is sufficiently great to eliminate the need for a statistical test for significant difference. These results cannot be due to volume differences since the second cleavage is an equal one. The average diameters, as measured with an ocular micrometer, of 75 anterior and 91 posterior blastomeres were 103 microns and 104 microns, respectively.

DISCUSSION

As shown in Table I, the ratios in enzyme activity between the blastomeres vary from 1.8 to 4.5. To what extent this range of values reflects experimental error

is difficult to determine. The data in Figure 1 give some indication of accuracy although not all these measurements were made consecutively. A test for reliability of the method gave very good results; thus, in a series of determinations using five eggs each time, the velocity constants were 0.083, 0.074, 0.073, 0.083, 0.076. Ultramicro methods are particularly subject to erratic and inexplicable errors, and the present method is no exception. One very probable source of error is the homogenization procedure which, due to the very small amounts of material, is difficult to standardize. These and other errors, however, tend to be eliminated by averaging the results of separate experiments, and it is felt that the average value in Table I is reasonably accurate.

These results have considerable value in support of the cytochemical studies which have been carried out on the ascidian egg. Ries (1937, 1939), Reverberi and Pitotti (1939), Urbani and Mistruzzi (1947), using the indophenol reaction, demonstrated a localization of indophenol blue oxidase (presumably cytochrome oxidase) in posterior blastomeres of various ascidian embryos. The present results are free from the objections which may be raised against cytochemical techniques; however, as it turns out, they offer a striking confirmation of the earlier cytochemical results.

The indophenol reaction has also been used with other types of eggs and embryos, principally by Child (1941) and his students. Thus differential appearance of color with the reagents has been demonstrated in sea urchin, amphibian, and chick embryos. The results presented here indicate that the use of this cytochemical test is valid, and that localization or gradients of cytochrome oxidase probably exist in these other forms.

As is known from studies on other types of cells, the cytochrome oxidase complex is located in mitochondria and presumably this is true in the *Ciona* egg. The localization of cytochrome oxidase in the particulate fraction of centrifuged homogenates of *Ciona* eggs and the action of sodium desoxycholate on crude homogenates lend support to this assumption. The results, therefore, indicate a marked localization of mitochondria in the posterior blastomeres.

From cell lineage studies of the ascidian egg it is known that the myoplasm, the region rich in cytochrome oxidase, gives rise to the muscles of the tadpole. Conklin (1931) demonstrated that displacement of the pigmented inclusions from the myoplasm of *Styela* eggs did not alter subsequent differentiation of muscles. Similarly located "mitochondria" in *Ciona* eggs were not essential for muscle formation. Although Conklin refers to these inclusions as mitochondria, he reserves the possibility that they may be lipid granules due to their staining properties and their low density as indicated by displacement to the centripetal end of centrifuged eggs.

There are indications that these granules lack cytochrome oxidase which is an additional reason for not referring to them as mitochondria. In centrifuged eggs the indophenol reaction is absent in the centripetal region (Ries, 1939; Urbani and Mistruzzi, 1947). Experiments carried out in this laboratory demonstrate that the cytochrome oxidase-containing elements have a high density. Egg homogenates (50 eggs in 5 μl. phosphate buffer) were fractionated by centrifugation and the cytochrome oxidase measured by the microspectrophotometric method. The light fraction composed of oil droplets and small granules, presumably the "mitochondria" described by Conklin, contained little or no cytochrome oxidase whereas

the heavier fraction contained nearly all the enzyme. In another experiment a solid mass of eggs (about 5 μl.) with no excess fluid was homogenized and fractionated by centrifugation. Samples of the light fraction consisting of oil droplets and small granules showed little cytochrome oxidase activity as compared to a high oxidase activity of the heavier fractions.

It seems reasonable to conclude that the inclusions which Conklin described in the myoplasm of the ascidian egg are not cytochrome oxidase-containing mitochondria and therefore their lack of influence on muscle differentiation has no bearing on whether a high oxidase activity is necessary for such differentiation.

Experiments which are more pertinent to this question are those of Ries (1939) who concluded that the cytochemically demonstrated oxidases were essential for normal differentiation of muscles. The region of the centrifuged egg which gave rise to muscles always coincided with that which gave a strong oxidase reaction. Furthermore if the reactive cytoplasm was displaced by centrifugation, the muscles differentiated in an abnormal region. In addition Reverberi and Pitotti (1939) relate the presence of the oxidases to normal cleavage and development of bilateral symmetry.

These authors, quite rightly, do not consider their experiments as indicating that oxidases are the primary cause of differentiation. It seems obvious that if there is a segregation during cleavage of particulate elements such as mitochondria, we cannot assume that our indicator of this segregation, such as cytochrome oxidase, is in itself the cause of differentiation. Particulate elements are complex in structure and function, all of which factors would have to be considered with respect to differentiation rather than a single component.

Mitochondria are generally considered to be the "power plants" of the cell and supply energy for cellular activities. The potential for energy production of posterior blastomeres might be related to subsequent differentiation, although at an early stage their energy requirement, as measured by oxygen consumption, cannot be more than 20% different from that of anterior blastomeres (Holter and Zeuthen, 1944). It is evident that more information is needed before we can be specific in relating differentiation to a localization of specific enzymes, or even, as the results suggest, a differential distribution of mitochondria.

Summary

Anterior and posterior blastomeres were separated in the four-cell stage of *Ciona intestinalis* and cytochrome oxidase activities of homogenates of the blastomeres were quantitatively determined by a microspectrophotometric method. The average cytochrome oxidase activity of homogenates of posterior blastomeres is 2.7 times that of anterior blastomeres. The results indicate a localization of mitochondria in the posterior blastomeres.

Literature Cited

Borei, H., 1950. Reactivation and protection of cytochrome oxidase preparations. *Biochem. J.*, **47**: 227–233.

Castle, W. E., 1896. The early embryology of *Ciona intestinalis*. *Bull. Mus. Zool., Harvard*, **27**: 203–280.

Child, C. M., 1941. Patterns and problems of development. University of Chicago Press, Chicago.

CONKLIN, E. G., 1931. The development of centrifuged eggs of ascidians. *J. Exp. Zool.*, **60**: 1–119.

COOPERSTEIN, S. J., AND A. LAZAROW, 1950. A microspectrophotometric method for the determination of cytochrome oxidase. *J. Biol. Chem.*, **189**: 665–670.

HESS, H. H., AND A. POPE, 1953. Ultramicrospectrophotometric determination of cytochrome oxidase for quantitative histochemistry. *J. Biol. Chem.*, **204**: 295–306.

HOLTER, H., AND E. ZEUTHEN, 1944. The respiration of the egg and embryo of the ascidian, *Ciona intestinalis*. *C. R. Lab. Carlsberg, Série Chim.*, **25**: 33–65.

LOWRY, O. H., AND O. A. BESSEY, 1946. The adaptation of the Beckman spectrophotometer to measurements on minute quantities of biological materials. *J. Biol. Chem.*, **163**: 633–639.

REVERBERI, G., AND M. PITOTTI, 1939. Differenziazione fisiologiche nell 'uovo delle Ascidie. *Comment. Pontif. Acad. Sci.*, **3**: 469–488.

RIES, E., 1937. Die Verteilung von Vitamin C, Glutathion, Benzidinperoxydase, Phenolase (Indophenolblau-oxidase) und Leukomethyleneblauoxydoredukase während der frühen Embryonalentwicklung verscheidener wirbelloser Tiere. *Pubbl. Staz. Zool. Napoli*, **16**: 363–401.

RIES, E., 1939. Versuche über die Bedeutung des Substanzmosaiks für die embryonale Gewebedifferenzierung bei Ascidien. *Arch. exp. Zellf.*, **23**: 95–121.

URBANI, E., AND L. MISTRUZZI, 1947. Le reazioni delle perossidasi, delle fenolasi e del glutathione studiate comparativamente nell 'uovo di alcune specie di Ascidie. *Pubbl. Staz. Zool. Napoli*, **21**: 67–80.

THE FAILURE OF HYPOPHYSECTOMIZED FUNDULUS HETEROCLITUS TO SURVIVE IN FRESH WATER

CHARLES E. BURDEN [1, 2]

The Bingham Oceanographic Laboratory, Yale University, New Haven, Connecticut

A pituitary control of osmoregulation in *Fundulus heteroclitus* was first suggested by Pickford (1953). She noted that six hypophysectomized fish which had been placed in fresh water were unable to survive, although such operated fish would live indefinitely in sea water and normal *Fundulus* will withstand abrupt changes of salinity with no adverse effects. These results were unexpected, since hypophysectomized *F. heteroclitus* (Matthews, 1933; Abramowitz, 1937), as well as similarly operated eels (Fontaine *et al.*, 1949; Callamand *et al.*, 1951), and certain strictly fresh water teleosts (*e.g.*, Abramowitz, 1937; Chavin, 1954) have been reported to survive in fresh water for considerable periods of time.

The problem of osmoregulation in teleostean fishes has been extensively studied and is reviewed by Baldwin (1937), Krogh (1939), Black (1951) and Fontaine (1953). Despite much research, a hormonal control of water and salt balance, such as is known in higher vertebrates, has not been conclusively demonstrated in teleosts. In the mammal, pituitary control of osmoregulation involves at least two antagonistic renal functions: the neurohypophyseal antidiuretic factor (vasopressin) acting directly on the kidney tubules (reviewed by Gaunt *et al.*, 1951) and the growth hormone (GH) acting apparently in conjunction with corticotropin (ACTH) and thyrotropin (TSH) through the adrenal cortex, to increase urine flow (reviewed by White, 1955). In amphibians the neurohypophysis regulates water metabolism by both renal and extrarenal mechanisms. Injections of neurohypophyseal hormones promote glomerular antidiuresis, and resorption of water by the kidney tubules and bladder (Sawyer, 1956). Water uptake, which takes place primarily through the skin, is increased by the injection of neurohypophyseal hormones. This response is diminished after hypophysectomy but may be restored to normal by a combination of ACTH and TSH (Levinsky and Sawyer, 1952).

Mammalian neurohypophyseal hormones appear to have no effect upon teleostean osmoregulation (Keys and Bateman, 1932; Burgess, Harvey and Marshall, 1933; Boyd and Dingwall, 1939; Fontaine and Raffy, 1950). Salmon posterior lobe is also ineffective (Callamand *et al.*, 1951). Nevertheless, Arvy *et al.* (1954, 1955) observed that there was a depletion of neurosecretion in the hypothalamo-hypophyseal system of *Phoxinus* and *Anguilla* after transfer to hypertonic media. In mammals it is well known that an increased salt load leads to a depletion of neurosecretory material, correlated with a decrease in the content of antidiuretic

[1] Supported by National Science Foundation Grant No. G941 under the direction of Dr. G. E. Pickford, principal investigator. This study fulfilled the requirements of an undergraduate research course for qualified students in the Zoology Department of Yale University.

[2] Present address: Vanderbilt Hall, Harvard Medical School, Boston 15, Massachusetts.

hormone in the hypothalamo-hypophyseal system (see Bargmann, 1954). The secretion of neurohypophyseal hormones in mammals apparently also stimulates the rapid release of corticotropin (Martini and Morpurgo, 1955) and thyrotropin (D'Angelo, 1955) in response to stress. Therefore the depletion following salt stress in fish does not necessarily signify an antidiuretic function of the neurohypophyseal hormone(s).

However, there is some evidence of hormonal control of water-electrolyte regulation in fish. Kalashnikov and Skadovskii (1948) observed lowered blood chloride and osmotic pressure following injections of hypophyseal extracts in the sturgeon. Smith (1956) found that salinity tolerance was increased in trout by treatment with growth hormone. Many investigators have presented evidence favoring greater thyroid activity in hypotonic than in hypertonic media in a number of teleostean species; this was recently demonstrated, for example, in *Fundulus* by Gorbman and Berg (1955), using radioactive iodine uptake studies. Fontaine (1956) notes that survival of a number of marine teleosts in fresh water may be significantly prolonged by administration of thyroxin or TSH before transfer. Chavin (1954) found a transitory stimulation of the adrenal cortical tissue of goldfish following saline immersion which he attributed to release of ACTH. Keys and Bateman (1932) showed that adrenalin enhances chloride secretion in the eel gill, but the relation of adrenalin secretion to the pituitary is not obvious.

Regardless of the hormones which may be involved, three sites of salt and water balance in teleosts may be affected. Although fish lack the dermal water balance mechanism found in amphibia, hypophysectomy could affect the permeability of the water barrier, thereby having a profound effect upon survival in non-isotonic media. A second site is obviously the kidney, which is actively concerned in water elimination in fresh water fish. Henschel (1934–35) found that the euryhaline plaice, but not the stenohaline sole, was able to increase urine flow upon transfer to fresh water. But it must be noted that on the basis of morphological studies Grafflin (1937) concluded that the failure of fresh water teleosts to adapt to sea water or *vice versa* must be attributed to extrarenal factors.

The salt-regulating function of the gills is well known. Considerable attention has been given to the cytological aspects of this mechanism. Chloride excretion was first attributed to large, acidophilic cells in the gill epithelium by Keys and Wilmer (1932). These "chloride cells" were found to be absent in the dogfish but abundant in six marine species of teleosts. In four fresh water forms the cells were infrequent or absent although acidophilic cells lacking contact with both the surface and the basement membrane were noted. Mucous cells were more abundant in the latter group.

Liu (1942) demonstrated that an enormous development of "chloride cells," previously hardly distinguishable in the gill of the paradise fish, *Macropodus opercularis*, could be brought about by gradually increasing the salt concentration of its environment. Copeland (1948), investigating the "chloride cells" of *Fundulus*, found that they characteristically possessed an open, distal vesicle in the sea water-adapted state which was lost, a clear protoplasmic area being found in its place, when the fish were transferred to fresh water. The critical salinity separating the salt and fresh water pictures was thought to lie between $\frac{1}{16}$ sea water and $\frac{1}{100}$ cold-blooded Ringers solution in tap water (Copeland, 1950).

Getman (1950) found the same cell type in *Anguilla rostrata* with far less marked cytological variation between fish adapted to fresh or salt water. He found that the vesicles or "pits" were less frequent, not absent, in fresh water adaptation, but noted that the results might be attributable to experimental conditions. Hoar (1951) mentioned the development of "chloride cells" at the time of parr-smolt transformation in the Atlantic salmon preparatory to seaward migration. Nishida (1953) found that "acidophilic great cells," which he identified with the "chloride cells" of Hoar and Copeland, were much more abundant and better developed in the smolt of the sea-run than the land-locked variety of *Onchorynchus masou* while both were still in fresh water.

The present investigation was concerned with a number of endocrinological and histological aspects of the problem in *Fundulus*. After confirmation of Pickford's findings regarding the loss of ability of hypophysectomized sea water-adapted *F. heteroclitus* to live in fresh water, hypophysectomy of fresh water-adapted fish was used to determine whether the impairment is that of survival or adaptation. Replacement therapy was employed to verify the fact that the observed condition is due to pituitary loss rather than to another factor inherent in extirpation of the gland, and an attempt was made to identify the pituitary fraction which enables the animal to live. In investigating the cause of death, studies of weight and blood chloride changes were made. Lastly, an examination of the gill cytology of the fish was carried out in search of clearly defined histological differences which might help explain the osmoregulatory difficulties.

I wish to express my sincere appreciation to Dr. Grace E. Pickford for suggesting the problem and for her guidance and encouragement. For making available the facilities of the Bingham Oceanographic Laboratory for these investigations I owe my thanks to its director, Dr. Daniel Merriman. I am indebted to other members of the laboratory staff; to Dr. Gordon A. Riley for determining water salinities, to Mr. Edward C. Migdalski for supplying the perch, and to the Misses Harriet A. Chambers, Rita M. Eurich, and Patricia J. Harris for their technical assistance and advice. I also thank Dr. Sanford L. Steelman of Armour and Company for the ACTH, TSH, and posterior lobe preparation, Dr. A. E. Wilhelmi at the Department of Biochemistry, Emory University, for the purified growth hormone, Dr. Tillman D. Gerlough of E. R. Squibb and Sons for the thyroxin, and Dr. Richard H. Barnes of Sharp and Dohme for the desoxycorticosterone acetate. Facilities for collecting sea water were provided by Dr. Victor L. Loosanoff, director of the U. S. Fish and Wildlife Service Laboratory at Milford, Connecticut. Finally, for translations of the Russian and Japanese literature employed I express my appreciation to Dr. Alexander Petrunkevitch and Mr. Mikiso Hane, respectively.

Materials and Methods

1. Running fresh water system

In order to test a relatively large number of hypophysectomized fish and controls under satisfactory conditions a running fresh water system was required. Such a system was necessary to avoid possible adverse effects from the handling involved in daily water changes or the definitely undesirable salt accumulation which would occur

in a standing tank from addition of food, evaporation, etc. As the only fresh water readily available was from the municipal water supply, three problems presented themselves in designing the required system: constant rate of flow, constant temperature, and dechlorination.

The arrangement is shown in Figure 1. Tap water was received into a constant level tank (A) placed above the aquaria. The water flowed from the tank by gravity at a rate regulated by a clamp (B) through a series of ten five-gallon carboys (C 1–10). The first two contained activated charcoal in sausage skins (D 1 and 2) to remove dissolved chlorine (modified method of Coventry, Shelford and

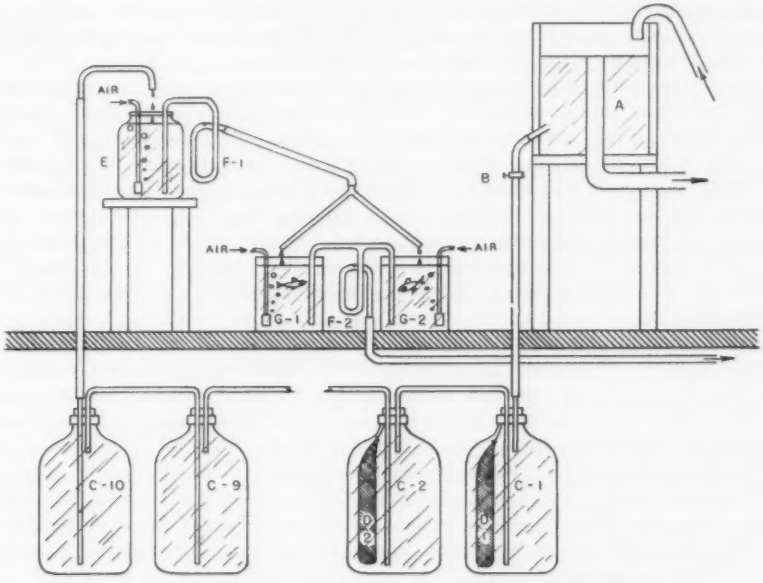

FIGURE 1. Running fresh water system (drawn by Patricia J. Harris).

Miller, 1935). The outflow from the last carboy was dripped into an aerated container (E) for the purpose of reoxygenation. From here water was fed into the aquaria (G 1 and 2) by means of a constant level siphon (F 1), a similar arrangement being used to adjust the outflow (F 2).

The flow of water was regulated to deliver ten gallons a day to each of two one-gallon aquaria, thereby giving a turnover rate of about once in every two and one-half hours. The battery of carboys created a reservoir such that it took over two days for water to pass through the system. During this time it reached aquarium room temperature which was regulated at *ca.* 15° C. Tests of the outflow with orthotolidine indicator showed it always to be chlorine-free. The only difficulty encountered was bubbles in the reservoir lines brought about by warming of the water. Flow irregularities due to these were easily avoided by removal of one of

the carboy stoppers for a few seconds once or twice daily. Five to seven fish could be maintained comfortably in each aquarium.

2. Experimental procedures

All fish used were *Fundulus heteroclitus* males and females 2¾ to 3¼ inches in length. Hypophysectomy, feeding, and illumination arrangements were the same as described by Pickford (1953). Operations and injections were made under anesthesia with tricaine methane sulfonate. Fresh rather than salt water was used exclusively when such procedures were being carried out with fish being maintained in the fresh water system. All experiments were made at 14–17° C. The pH of the sea water from the storage tank and in freshly diluted mixtures was *ca.* 8.5. However, in long established sea water tanks where the fish were usually held it fell to *ca.* 7.4. The pH of the fresh water ranged from 7.2 to 7.6.

One or two unoperated controls were kept in the aquaria with each experimental group, making a total of about 30 normal fish being maintained in fresh water for periods of two weeks to three months each. All of these fish remained healthy, although, as also in the case of fish in salt water, it was occasionally necessary to treat them for minor skin infections, either with aureomycin or malachite green as recommended by Pickford (1954a). Hypophysectomized fish in salt water rarely die as an immediate result of the operation but a small number of fish, of the order of 5–10%, were lost at later dates from causes other than the experimental treatments. In all a total of about 100 hypophysectomized fish were employed in the experiments.

(*a*) *Tests on survival of "long term" hypophysectomized sea water-adapted fish in fresh water.* A group of about 60 fish that had been kept in sea water were hypophysectomized and returned to this medium. They were allowed to recover for at least two weeks and were then tested for their ability to survive in fresh water. Out of 56 so tested only three were able to live indefinitely, and these showed positive evidence of incomplete hypophysectomy (growth in length and, in males, development of nuptial coloration); they were therefore excluded from the experiment. The remaining 53 fish developed symptoms of asthenia, described below, but 39 of them were saved by gradual return to sea water through 1/10, ¼, and ½ dilutions over a period of about 24 hours. Fish that recovered were kept under observation until they were again eating normally and then were allowed at least a few extra days before being used in other experiments. The average time for complete recovery was about two weeks.

(*b*) *Tests on survival of "long term" hypophysectomized sea water-adapted fish in diluted sea water.* These experiments were limited to a small number of previously untested fish and are of a preliminary nature. The tests were carried out in similar aquaria to those used in the fresh water system, the water being changed two or three times weekly. Two hypophysectomized fish and one unoperated control were tested in 1/10 and ¼ sea water (*ca.* 2.6 and 6.5 ‰, respectively); four hypophysectomized fish and one control were tested in ½ sea water (*ca.* 13 ‰).

(*c*) *Tests on ability of fresh water-adapted fish to survive hypophysectomy in fresh water.* A group of 25 fish were kept in fresh water for about four weeks before operation and were returned to this medium after removal of the pituitary. Survival times were studied as in the preceding groups and those that recovered in sea water were saved for other experiments.

(*d*) *Tests on ability of sea water-adapted fish to survive transfer to fresh water immediately after hypophysectomy.* A small group of eight sea water-adapted fish were transferred to fresh water immediately after hypophysectomy. These served as controls for the group that had been pre-adapted to fresh water.

(*e*) *Weight changes in fresh and salt water.* Two series of experiments were made in each medium with four previously untested, hypophysectomized fish in each group. One series was weighed daily until onset of asthenia in the fresh water group and for a comparable time in the sea water group. A second series was treated similarly but received only initial and final weighings. Two unoperated controls were included with each group, except those receiving initial and final weighings in sea water, making a total of 22 fish employed in the experiment. Weighings were made on a triple beam balance following anesthesia and extremely light blotting. It is realized that the small numbers employed detract from the significance of this experiment but time did not permit further study and the results appear to be worth reporting.

(*f*) *Blood chloride determinations.* Blood chlorides were determined by a microadaptation of the method of Schales and Schales (Hawk, Oser and Summerson, 1947). Blood serum samples were collected from normal fish adapted to salt or fresh water (8 days) and from hypophysectomized fish adapted to sea water and on the verge of death in fresh water (4–7 days). Whole blood was obtained by cutting off the tail and allowing the blood to drop on to a paraffined spot plate. Ten- to 30-mm.^{3} samples were centrifuged in an air driven "spinning top" rotor (Glick, 1949) and 4.8-mm.^{3} quota of serum were transferred into titration vessels with the aid of a constriction pipette. Before titration two magnetic beads and *ca.* 45 mm.^{3} of water containing 0.06 ml. of indicator solution per 2 ml. were added to the sample. Titration was made without deproteinization using a 70-mm.^{3} microburette and magnetic stirrer.

(*g*) *Hormone therapy.* These experiments were all made on hypophysectomized fish that had been previously tested for their inability to survive in fresh water and had subsequently been allowed to recover in sea water.

Fourteen series of intraperitoneal injections were made in an attempt to determine what treatment would enable hypophysectomized *Fundulus* to survive in fresh water. Breis of *F. heteroclitus, Pollachius virens,* and *Perca flavescens* pituitaries, in addition to solutions or suspensions of ACTH, desoxycorticosterone acetate (DOCA), TSH, thyroxin, GH, posterior lobe preparation, a combination of ACTH, TSH, and GH, and three series of saline controls, were employed. The breis and hormones were made up in 0.6% NaCl. Unless otherwise noted, groups of four fish were given daily injections of 0.005 ml. per gram weight on alternate days with four injections (one week) in sea water before transfer to fresh water.

Fundulus pituitary glands were collected from fish weighing from seven to 40 grams, caught near New Haven just prior to spawning in April, 1955. Two hundred glands, averaging slightly under one milligram each in weight, were contained in each milliliter of homogenate so that each fish received one gland per gram weight at each injection. Fresh frozen glands from the stenohaline marine pollack were collected from prespawning fish during the summer of 1954, at Wilson's Beach, Campobello Island, by G. E. Pickford and assistants. The brei was prepared in about the same concentration by weight as the *Fundulus* material, 200 mg./ml. Two series of experiments were carried out using this preparation. The first was

under the same conditions as with the *Fundulus* brei. The second series, comprising two hypophysectomized fish and one unoperated control, was given daily treatment with the homogenate at 0.01 ml. per gram and with but two injections before transfer to fresh water. Thus they received four times as much material as did the first series. The average weight of the pituitary of post-spawning yellow perch weighing from 30 to 100 grams each was found to be of the same order as that of pre-spawning *Fundulus* weighing 7–40 grams, *ca.* 1 mg., and hence the same concentration (200 glands/ml.) was used. Post-spawning fish, caught during June–July, 1955, in the New Haven area, were employed.

ACTH dosage (Armour's Acthar Lot No. R-491284-U, 0.05 USP units/gm.) was based on that used by Pickford (personal communication) who obtained definite stimulation (increased cell layers, proliferation) of the interrenals of *F. heteroclitus* within one week. Desoxycorticosterone acetate injections (Sharp and Dohme Lot HXVII-06-07-4-1, 25 μg/gm.) were equivalent to those given by Kirshenblatt (1952) to vy'un (*Misgurnus fossilis*) in which he was able to induce egg maturation and ovulation. On the basis of the thyroid stimulation findings of Pickford (1954b) 1 μg/gm. was selected for injections of TSH (Armour Lot No. 317–51, 1.2 USP/mg.). About 5 mm.^{3} 0.1 N NaOH/ml. was required to bring this into solution. Since there is no established function in fish on which to base dosage, thyroxin (Squibb BT00080) was somewhat arbitrarily set at 2 μg per gram weight. It is of the same order as that used by many investigators on fish and has been shown to increase resistance to skin infections in *Fundulus* (Pickford, unpublished observations). Addition of 20 mm.^{3} 0.1 N NaOH/ml. was required to dissolve the hormone. In these two series, which were made early in the investigation, before the effects of *Fundulus* brei had been established, only two injections in sea water prior to fresh water transfer were made and injections were given daily.

Growth hormone dosage (Wilhelmi Beef B168GH, 10 μg/gm.) was based on that given by Pickford (1954a) by which she obtained growth of the same order as occurs in normal fish. Ten mm.^{3} 0.1 N NaOH/ml. were required for solution. Injections of whole posterior lobe preparation (Armour Hog Lot No. 503, 100 mU/gm.) were given at twice the dosage determined as one *Fundulus* unit in spawning reflex assays (Wilhelmi, Pickford and Sawyer, 1955). The concentration of each hormone in the GH-TSH-ACTH combination remained the same as when used separately: 10 USP units ACTH, 2 mg. TSH (Armour Lot No. 2R3, 0.4 USP/mg., less potent then 317–51 but still at a higher dosage than that used effectively by Pickford, 1954b), 10 mg. GH, 15 mm.^{3} 0.1 N NaOH/ml. Of three series of saline controls, the first received injections of 0.6% NaCl under the usual conditions. A second and a third group, each with two hypophysectomized fish and an unoperated control, were injected daily with 0.6% and 0.06% NaCl. No build-up injections in sea water were made with these last two series.

(*h*) *Histological study.* The thyroids and the interrenals of the group receiving *Fundulus* brei were treated as previously described by Pickford (1953). Hemoglobin was determined with the aid of a Tallqvist-Adams scale. The study of the gill cytology was carried out according to the Regaud-Altmann mitochondrial technique recommended by Copeland (1948). Experience proved that best results could be obtained if the picric acid differentiating fluid were used merely to wash off excess acid fuchsin after cooling and differentiation were accomplished by flooding the slide with 1% aqueous methyl green for about 5–10 seconds. It is worth

noting that the heating of Altmann-flooded slides should be stopped when white fumes are first evoked.

Gills of four normal sea water-adapted *Fundulus*, five normal fresh water-adapted specimens, six hypophysectomized fish which were sea water-adapted, four hypophysectomized *F. heteroclitus* on the point of death in fresh water, and those of the series receiving *Fundulus* brei were selected for study. Each set was also stained with hematoxylin and eosin and by the azan method for confirmatory studies.

Results

(*a*). Of the total of 53 long-term hypophysectomized fish tested by transfer to fresh water, 14 were found dead or so nearly so as not to recover subsequently.

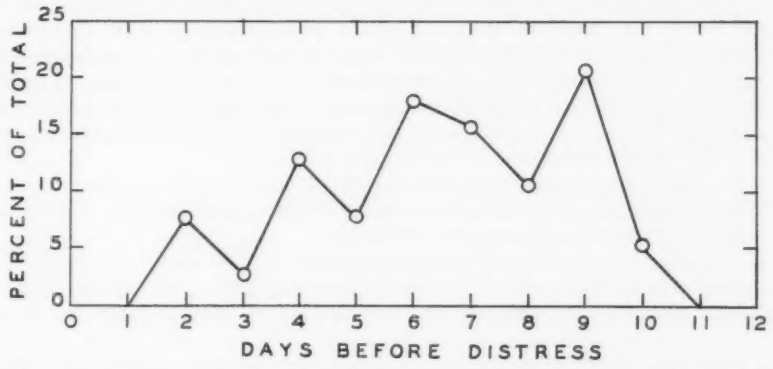

Figure 2. Time of onset of asthenia in 39 sea water-adapted "long term" hypophysectomized *F. heteroclitus* after transfer to fresh water. Only fish that were saved and recovered in sea water are used (see text).

The 39 remaining fish, removed when found lying on their sides, recovered completely after being transferred to sea water. These latter fish and the number of days they remained in fresh water before removal was necessary, are shown graphically in Figure 2. Only these 39 are considered since it is felt that subsequent recovery in sea water is the most conclusive demonstration that the fresh water was the sole cause of their distress.

The following symptoms preceded death in fresh water: The fish refused to eat after the first or, occasionally, the second day. After about two thirds of the total time which elapsed before death they became extremely quiescent and stayed on the bottom of the aquarium. About a day before death the fish were noticeably tilted and would be found lying completely on one side twelve hours later, usually ventilating their gills rather rapidly. This was the point at which there could be no doubt that the fish were succumbing and this stage was selected for rescue for use in subsequent experiments. Shortly before death they would be found completely upside down and frequently could be saved even in this state. Return to sea water was accompanied by hydrostatic and equilibrium difficulties far exceeding those experienced by normal fish.

TABLE I

Time of onset of severe asthenia in "long term" hypophysectomized, sea water-adapted F. heteroclitus in different dilutions of sea water

Per cent sea water	Number of fish	Days				
		5–10	11–15	16–20	21–25	26–30
10%	2	—	2	—	—	—
25	2	—	—	2	—	—
50	4	1	—	1	—	2

Time did not permit study of the ability of these fish to withstand a second transfer to fresh water, since the tested fish were needed for hormone therapy. Such a project would have required the study of the entire group, in order that possible differences in mean survival time would be statistically significant, and this would have greatly curtailed the study of other aspects of the problem. For replacement therapy experiments it appeared more important to use saline-injected controls since it is known that such treatment tends to increase the weight loss and lower the viability of hypophysectomized *F. heteroclitus* in salt water.

(*b*). Table I summarizes the results for fish tested in diluted sea water. At all three dilutions the normal controls were still active and eating long after their hypophysectomized counterparts had succumbed.

(*c and d*). The results obtained by operating on fresh water-adapted fish and placing sea water-adapted fish in fresh water immediately after the operation are graphed in Figure 3. Owing to the small number of cases in these two groups,

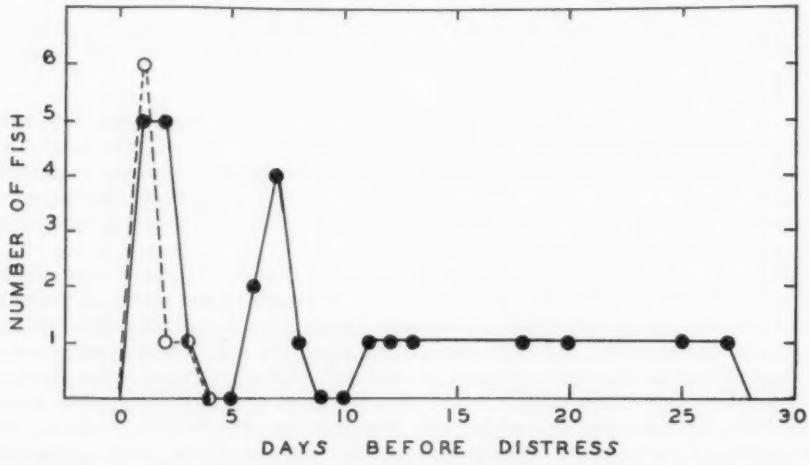

FIGURE 3. Time of onset of asthenia or death in hypophysectomized *F. heteroclitus* placed in fresh water immediately following the operation. Solid line represents 25 fish previously fresh water-adapted. Broken line indicates the eight previously sea water-adapted.

TABLE II

Weight change tests

Group		Days	No. of fish	Mean weight change	*s*
Daily weighings:					
Fresh water	Hypects	2–3	4	13.8	3.4
	Controls	2	2	−0.1	—
Sea water	Hypects	3	4	−4.0	3.2
	Controls	3	2	−6.5	—
Two weighings only:					
Fresh water	Hypects	6–9	4	4.3	4.5
	Controls	10	2	7.0	—
Sea water	Hypects	6	4	−4.1	5.0

those which did not recover subsequently are included as well. Out of the group of 25 fresh water-adapted fish, 11 succumbed during the first three days, seven survived 6–8 days, and another seven survived 11–27 days. In the second group, all died in the first three days, six after one day, and one on each of the following two days.

(*e*). Table II contains the results of the weight change tests. Hypophysectomized fish succumbed more rapidly in fresh water when handled daily than when weighed only at the beginning and end of the experiment, 2–3 days as compared with 6–9 for the latter group. Both hypophysectomized groups showed a slight weight increment at the time of onset of asthenia in fresh water, greater in the case of daily handling (13.8% versus 4.3%). Hypophysectomized fish in sea water showed a small decline (*ca.* 4%), irrespective of whether they were handled daily or not. The number of controls is too small in each group, but appears to indicate that daily handling causes weight loss in sea water but little change in fresh water. A mean weight increment in unhandled fish in fresh water is probably due to natural growth, since a period of 10 days elapsed.

(*f*). In Table III blood chloride determinations are given. Normal fish in either sea water or fresh water maintain a constant blood chloride level (0.885–0.887) and the blood chloride of hypophysectomized fish in sea water is not sig-

TABLE III

Blood chloride determinations

Group	Number of fish	Serum NaCl, gm.%		
		Range	Mean	*s*
F.W. Hypects	6*	.322–.451	.383	.049
F.W. Normals	6	.809–.996	.887	.078
S.W. Hypects	5	.777–.872	.817	.036
S.W. Normals	5	.818–.957	.885	.051

* Samples were taken at the onset of acute symptoms. Three of the fish in this group had been receiving GH-TSH-ACTH and DOCA injections.

nificantly lower ($P = 0.05$–0.1). When hypophysectomized fish are placed in fresh water, however, a blood chloride averaging only 43% of normal is found when asthenia begins.

(g). The results of injection therapy are found in Table IV. The fish's numbers are given so that it can be seen which ones were used in more than one series and their performance in each compared. Those receiving *Fundulus* brei were still active and eating after 20 days in fresh water. At that time it was decided that the preparation was undoubtedly enabling them to survive, so the fish were killed for autopsy. Numerous fish being tested for fresh water survival for the first time or receiving injections of other preparations succumbed during this 20-day period.

The results of the autopsy of the fish receiving *Fundulus* brei (Table V) indi-

TABLE IV

Results of injection therapy

Material injected	Fish number	Days in fresh water		Subsequent recovery completed (days)
		Previous test	With injections	
Fundulus brei†	4	7	20	(Discontinued)
	25	(*)	20	(Discontinued)
	55	6	20	(Discontinued)
	107	9	20	(Discontinued)
Perch brei†	68	10	6	Died next day
	58	8	8	3
	B108	(*)	16	Found dead
	G3	6	19	(Discontinued)
Pollock brei (Alternate days)	14	6	2	5
	77	9	3	3
	68	10	5	4
	58	8	7	3
Pollock brei (Daily)	12	4	4	Died 3 days later
	11	3	6	Died 2 days later
ACTH	76	6	3	Found dead
	44	(*)	5	4
	78	7	5	3
	32	(*)	5	3
DOCA	44	(*)	2	Found dead
	14	6	2	2
	61	7	2	Died 16 days later
	32	(*)	4	Found dead
	G25	10	5	Blood sample taken

* Fish tested by use in weight change test or hypophysectomized after fresh water adaptation and hence not comparable.

† In each of these groups a fifth fish died under anesthesia after the fourth (perch) or fifth (*Fundulus*) injection. These are most unusual circumstances not otherwise encountered in many hundreds of injections.

TABLE IV—*Continued*

Material injected	Fish number	Days in fresh water		Subsequent recovery completed (days)
		Previous test	With injections	
TSH	21	6	3	Died next day
	18	(*)	4	6
	17	(*)	5	6
	24	6	5	4
Thyroxin	4	7	3	13
	26	5	3	16
	9	6	5	Died 20 days later
	2	4	6	5
GH	44	(*)	4	4
	77	9	4	4
	G3	6	5	5
	68	10	5	4
GH-TSH-ACTH	G13	9	2	Found dead
	G29	9	3	Found dead
	G26	5	3	3
	G30	7	4	Blood sample taken
	46	3	4	Blood sample taken
Posterior lobe preparation	32	(*)	3	4
	14	6	4	5
	78	7	4	4
	G23	7	9	Died next day
0.6% NaCl (Alternate days)	47	4	2	Found dead
	G12	5	3	Found dead
	96	8	2	Died 8 days later
	46	3	5	5
0.6% NaCl (Daily)	11	3	2	3
	9	6	4	6
0.06% NaCl (Daily)	8	4	3	10
	2	4	4	5

cate moderate to strong stimulation of the thyroid and interrenal. Sexual maturation was nearly as great as in normal prespawning *Fundulus*. The experiment was of too short duration to evaluate growth, but length increments in two of the fish indicate the presence of growth hormone, despite the small average weight loss, probably attributable to multiple effects of treatment. The anemia of hypophysectomy was not alleviated.

Injections of pollock brei failed to demonstrate any salutary effect. Nuptial colors, indicating the presence of gonadotropins, appeared in some males during the 2–7 day period before onset of acute symptoms, but since test of subsequent recovery in sea water seemed important, autopsy was not performed to check the presence

of other hormones. Due to time limitations the experiment employing the perch pituitary brei was discontinued before the full 20 days, although one fish was still showing none of the symptoms preceding death in fresh water at that time.

The daily pollock brei treatment given to a normal fish in fresh water over a twelve-day period gave evidence of no adverse effects. The saline controls were also unaffected by the injections received. Yet, except for the perch and *Fundulus* breis, none of the injections gave indications of increasing the ability of the hypophysectomized fish to survive in fresh water.

TABLE V

Condition of fish receiving Fundulus brei at the time of autopsy compared with normal values. (GSI = Gonad Wt. × 100/Body Wt.; HSI = Liver Wt. × 100/Body Wt.; TEH = Average thyroid cell height in microns)

Fish number and sex	Wt. change %	Length change %	GSI	HSI	Anterior interrenal	TEH	Hemoglobin
Fundulus brei:							
4 ♂	− 2.1	−0.8	3.9	3.9	Hypertrophy	5.0	45
55 ♂	−10.1	1.4	6.9	5.1	Hypertrophy	14.4	55
107 ♂	1.5	0	3.1	4.8	Hypertrophy	12.4	50
25 ♀	− 1.3	1.7	4.4	5.2	Hypertrophy	6.0	45
Hypect controls:*							
♂ ♂	− 1.2	−0.2	0.3	5.4	Inactive but not atrophied	3.8	68
♀ ♀	−10.4	0	1.1	6.4	Inactive but not atrophied	—	54
Normal wild fish:**							
♂ ♂ Nov.	—	—	0.7	6.3	—	6.5	—
May	—	—	5.8	2.8	—	6.9	80
♀ ♀ Nov.	—	—	1.5	6.4	—	6.0	—
May	—	—	6.1	4.8	—	5.9	—

* Males (Pickford, 1954b), means of five fish saline-injected thrice weekly for one month in sea water. Females (Pickford, unpublished) means of three saline-injected daily for one week in sea water.

** Means of about 40 fish per group ♂ ♂ (Pickford, 1953), ♀ ♀ (Pickford, unpublished), taken from sea water.

(*h*). In the histological study, the sea water-adapted fish were found to have a gill cytology essentially as described by Copeland (1948): the chloride cells were columnar, acidophilic, reaching from the free surface to the basement membrane, and usually with an "excretory vesicle" at the distal border. In normal fish adapted to fresh water for a week or more, however, most of the cells exhibited shrinkage, rounding up, and frequent loss of contact with the free surface; they were also far less fuchsinophilic. Typical sea water-adapted cells, sometimes with vesicles, were noted, but less commonly. In hypophysectomized fish in fresh or sea water the chloride cells were cytologically the same as normal fish in these respective media.

A cursory count of the mucous cells revealed an average of about four per section in the sea water-adapted and eight in the fresh water-adapted normal fish.

The mucous cells of hypophysectomized fish were emptied and atrophied in contrast to the normal condition. In addition, although the sea water-adapted fish had about four mucous cells per filament section, as did their unoperated counterparts, in hypophysectomized fish in fresh water the number was about two per section, much less than normal. Hypophysectomized fish receiving *Fundulus* brei in fresh water showed the unoperated fresh water-adapted picture; the mucous cells appeared full, well stained, and averaged seven to eight per section.

Discussion

1. Survival in fresh water

There is no doubt that untreated hypophysectomized *Fundulus heteroclitus* cannot withstand transfer to fresh water under the conditions of these experiments. Many fish were saved and used again for unsuccessful injection therapy, injections which apparently have no adverse effects upon hypophysectomized fish in sea water or normal fish in either medium. Adding these 46 instances in which the fish were retested and still failed to survive to the 53 of the first test, a total of 99 cases are available to support this thesis. The more than thirty normal fish, unaffected by transfer from salt to fresh water at various times in company with the experimentals, exclude the possibility of harmful factors in the running fresh water system.

It would seem from the survival time (Fig. 2), varying between two and 10 days with an average of about 6½ days, that the cause of distress is slow in reaching its full effects. The form of the curve, rising slowly and dropping suddenly, supports this contention. It is highly unlikely that the irregularity of the curves derives from any other cause than lack of sufficient numbers to smooth it out.

The wide range of survival times obtained by operating on fresh water-adapted fish appears to show three asthenia peaks (Fig. 3). Out of the group of 25 fish, 44% succumbed during the first three days, 28% survived 6–8 days, and another 28% survived for 11–27 days. These results may be compared with the incidence of asthenia in sea water-adapted fish transferred to fresh water two or more weeks after hypophysectomy (Fig. 2). In this group there was a single peak at 6–7 days, only 10.3% died during the first three days, and none survived longer than 10 days. On the other hand, when sea water-adapted fish are transferred to fresh water immediately after removal of the pituitary they succumb rapidly (Fig. 3, broken line); out of a group of eight such fish none survived more than three days, although it is extremely rare for hypophysectomized fish to die (except later, from collateral effects such as the development of kidney stones, Pickford, 1953) when they are returned to sea water. The survival time in fresh water-adapted *Fundulus* therefore appears to be the result of two opposing factors: an increased sensitivity to fresh water immediately after the operation, and, if this period is withstood, a tendency for the prolongation of survival which could be attributed to pre-existing fresh water adaptation.

The results of Abramowitz (1937) and Matthews (1933), referred to in the introduction, remain unexplained. But the survival time which they noted ("several weeks") is not far outside the limit for fish hypophysectomized in fresh water reported herein. Even with normal *Fundulus* there has been a discrepancy between the results of different authors (Garrey, 1905; Sumner, 1906).

2. Cause of death

The low blood chlorides of hypophysectomized fish dying in fresh water (Table IV), less than half of that of operated fish in sea water or normal fish in either medium, is seemingly sufficient cause for death. These studies indicate that *Fundulus* normally has remarkable osmoregularity capacity, no difference being discernible in the blood chlorides of fresh and salt water-adapted fish. Rather than having a slightly higher blood chloride, resulting from decreased osmoregulatory capacity, the hypophysectomized fish in sea water appears to have a somewhat lower blood chloride although the difference is not quite statistically significant ($P = 0.05$–0.1).

Examples of serum chloride determinations on various fish are presented for comparison in Table VI. It is well known that the osmotic pressure of the blood of stenohaline marine teleosts is higher than that of fresh water species, and that euryhaline species are capable of a considerable degree of regulation. The change in blood chlorides is even greater than the fall in osmotic pressure accompanying

TABLE VI

Comparative data on blood chloride for fresh water, sea water and euryhaline teleosts

Author	Species	Serum or plasma NaCl in gm. per cent	
		F.W.	S.W.
Strictly fresh water:			
Vars (1934)	*Cyprinus carpio*	av. 0.477	—
	Esox lucius	av. 0.487	—
Puschel (1928)	*Tinca tinca*	av. 0.517	—
Drilhon (1942)	*Cyprinus carpio*	av. 0.626	—
Stenohaline marine:			
Hall, Grey, and Lepkovsky (1926)	*Brevoortia tyrannus*	—	0.862
Grafflin (1935)	13 species	—	0.875–1.008
Boucher-Firly (1935)	*Conger conger*	—	0.994–1.097
	Muraena helena	—	1.035–1.228
Euryhaline poikilo-osmotic:‡			
Boucher-Firly (1935),	*Anguilla anguilla*		
Callamand (1943), and	immature	0.375–0.701	0.661–1.170
Callamand *et al.* (1951)	hypophysectomized	—	0.631–0.769
	silver, migrating	0.155–0.491*	0.826–1.338
Euryhaline homio-osmotic:			
Koch and Heuts (1942)	*Gasterosteus aculeatus*		
	immature**	0.490–0.650	0.510–0.620
Present paper	*Fundulus heteroclitus*		
	normal	av. 0.887	av. 0.885
	hypophysectomized	av. 0.385†	av. 0.817

* Asthenia is associated with hypochloremia. In a later paper (Fontaine and Callamand, 1948) higher values for some silver eels are given.

** Sexually mature sticklebacks lose their ability to withstand higher salinities.

† Asthenic and on the verge of death.

‡ Osmotic pressure data, summarized by Black (1951) and Kalashnikov (1939), indicate that *Salmo trutta*, *S. salar* and *Acipenser stellatus* belong in this group.

transfer to lower salinities because of the concurrent increase in alkaline reserve (Fontaine and Boucher-Firly, 1934). Two types of euryhalinity are found in fish. In the first, exemplified by the eel, the blood chloride may fluctuate considerably with the environment, whereas in the stickleback and killifish it remains constant. It will be noted that in the hypophysectomized eel, as well as *Fundulus*, the blood chloride of hypophysectomized fish in sea water tends to be lower than normal. The generally lower blood chloride of *Anguilla* in fresh water is undoubtedly related to the fact that this fish is not capable of absorbing chlorides through the gills.

The proportionately small weight changes (Table III) indicate that the salt-regulating mechanism rather than failure of ability to excrete excess water is at fault. In addition, the fish showed no signs of edema such as would result from internal flooding.

The results of the tests in diluted sea water are surprising. The salinities of the $\frac{1}{10}$ and $\frac{1}{4}$ dilutions are below that of *Fundulus* blood. The results show, as might be expected, that survival is merely prolonged. The half and half dilution, equivalent to 1.30 gm. per cent NaCl, is considerably above the salinity of the blood, yet the fish did not survive indefinitely in this medium. The present investigation throws no light on the problem posed by this evidence.

The incidence of renal calculi was not studied. This trouble develops slowly over a period of three weeks or longer and is sporadic (Pickford, 1953). It can be excluded as a cause of death in fresh water since fish operated in fresh water also failed to survive long enough (except in a few instances) for renal lethiasis to develop. One of the four receiving *Fundulus* brei (No. 25) had calculi, seen in sections of the head kidney, yet survived as well as the rest.

3. Hormone therapy

Although there were only four cases, owing to limitations set by the difficulties involved in obtaining a large supply of *Fundulus* glands, it is clear that these injections were responsible for the long survival of the fish in fresh water. The experiment seems to have been adequately controlled to warrant acceptance of this fact: 1) the fish were pretested, 2) they were allowed to stay in fresh water twice as long as the maximum survival time for non-injected sea water-hypophysectomized fish and were still in good health at the end of that time, 3) controls receiving saline under identical conditions showed no increased survivability, and 4) untreated hypophysectomized fish kept in the aquarium at the same time succumbed as usual. It therefore appears safe to conclude that the *Fundulus* pituitary secretes a hormone or hormones enabling the fish to survive in fresh water.

Since all target organs of the adenohypophysis appear to have been stimulated by the *Fundulus* brei, the evidence from the autopsy throws no light on the nature of the particular factor involved.

Since two of the fish receiving perch pituitary brei survived in fresh water considerably longer than the established maximum for hypophysectomized *Fundulus*, it also seems most probable that the factor enabling the fish to live was present in this preparation. The fact that perch brei was not as successful as the *Fundulus* brei could be due to suboptimal amounts of the factor, wrong proportions in the event that more than one hormone is involved, or adverse foreign protein reactions.

Pollock pituitary, whether given under the same conditions or in quadruple

amounts (double dosage daily), did not aid survival. This preparation, therefore, either lacks the necessary factor altogether or one of the adverse factors mentioned as possibly present in the perch brei holds an even greater sway in the pollack preparation.

There is every indication that ACTH, TSH, GH, posterior lobe preparation, DOCA, and thyroxin alone or the first three in combination have no salutatory effect. Again it is possible that the dosages were too high or too low, but those of the first four were known to have a moderate influence on target organs. The conditions of injection are even less likely to be at fault. Most were given exactly as the successful *Fundulus* brei. Daily injections may be disturbing since daily weight change tests, not reported here, indicated that such handling was highly undesirable. However, in the presence of the necessary factor for survival it would seem that the hypophysectomized fish could overcome this difficulty, since daily handling does not seriously disturb normal fish in fresh water. Moreover, daily injections would seem essential for thyroxin, which presumably does not act through a target organ. The negative results with all these preparations are surprising in view of the literature which implicates one or the other in teleostean osmoregulation. The results with the posterior lobe preparation serve to substantiate previous findings—that these hormones apparently have no osmoregulatory effect in fish.

All the possibilities have not been exhausted in the tests with these hormones. Dosage levels have not been explored. Three well known vertebrate hormones, prolactin, gonadotropin, and intermedin, remain untested. There is no reason to suspect intermedin, which undoubtedly was present as a contaminant in many of the preparations tested. Gonadotropins also seem unlikely since immature killifish withstand transfer to fresh water as well as mature fish of either sex. In addition, both the pollock and perch breis contained relatively high amounts of the gonad-stimulating hormone and only one of these was successful, whereas the perch brei, evidently with low gonadotropic activity, had a salutatory effect. Prolactin has no known function in fish. Although an osmoregulatory control would not be suspected of the hormone from its known activity in higher vertebrates, it is not ruled out. More likely, however, since the pituitary breis of *Fundulus* and the strictly fresh water perch possess the factor, whereas it is probably absent in the marine pollock, an as yet undescribed hormone is involved.

4. Gill cytology

The cytological picture of the gills of normal *Fundulus* adapted to sea water was found to be exactly as that described by Copeland (1948) and requires no further comment. The results for fresh water adaptation obtained in this study more closely resemble the condition described by Copeland for fish in distilled water than his description of the condition in tap water. The discrepancy could be due to differences in the calcium content of the water or a similar cause which could be brought out only by complete tap water analyses. There is no doubt that the chloride cells are shrunken and less strongly fuchsinophilic in fresh water. When gills of fish adapted to each of the two media were stained on the same slide by the Altmann method, the chloride cells of the fresh water specimen were scarcely to be seen, while the sea water sections were excellent.

The fresh water cytological picture noted in this study is not unlike that described by Keys and Wilmer (1932), who found the chloride-secreting cell-type less abundant or absent in fresh water fish as compared with marine forms, while some acidophils were also seen rounded up below the epithelium in the former. The findings herein are also reminiscent of the hardly distinguishable cell in *Macropodus* before saline adaptation (Liu, 1942), the merely lessened abundance of chloride cells with vesicle noted by Getman (1950) in the eel, and the lesser chloride cell development noted by Nishida (1953) in the land-locked as compared with the sea-run salmon.

The fact that the chloride cells appear normal in hypophysectomized sea water-adapted fish is in keeping with the chloride excretion function, long attributed to these cells, since blood-chloride studies indicate that the fish maintain osmotic equilibrium perfectly well in this hypertonic environment. The obvious change in these cells after transfer to fresh water in normal fish was again found in hypophysectomized ones, indicating normal response as far as cutting down of chloride excretion is concerned.

Mucous cells are apparently holocrine. These are the only cells in the mammalian gastric mucosa which have a significant mitotic rate, presumably to replace cells extruded into the cavity of the gut (Stevens and Leblond, 1953). However, observations on the digestive system of hypophysectomized rats (Baker and Abrams, 1955) showed that the mucous cells are not affected. Nevertheless it appears that those in the gills of hypophysectomized *Fundulus* have discharged their contents and have not been replaced. Mucous cells may play a special role in fresh water fish. They were noted to be more predominant in the gills of fresh than in sea water-adapted fish in this investigation, and Keys and Wilmer found them so in fresh water over marine teleosts. The skin was not studied in the present investigation, but it appears likely that all epidermal mucus secretion would be under pituitary control if such is the case in the gills.

The protective role of mucus in the osmoregulation of fishes is well known. Firly (1932) found that removal of mucus from the skin by washing was more harmful to eels transferred from fresh to salt water than *vice versa*. The opposite effect was observed in this study; however, isotonic and hypotonic media were found to be deleterious, whereas the fish apparently maintain themselves well in hypertonic sea water. It appears obvious from the weight change and blood chloride studies that hypophysectomized *F. heteroclitus* in fresh water are able to cope with the increased water load imposed by the medium but suffer from an inability to maintain the normal blood chloride level. Since the site of chloride loss, whether through the gills, skin, or kidney is unknown, it is not possible to state definitely that atrophy of the mucous cells is involved. An increased permeability of the skin, resulting from lack of a protective coating, would lead to polyuria (Grafflin, 1931) with possible abnormal loss of chloride. But the failure to survive, even in an approximately isotonic medium, indicates that other factors are operative.

Summary

1. Hypophysectomy results in a loss of ability to survive in fresh water by the euryhaline cyprinodont, *Fundulus heteroclitus*. The average survival time is 6–7

days for salt water-adapted fish. Preadaptation in fresh water before operation may prolong survival but does not prevent ultimate death.

2. Hypophysectomized fish are unable to survive in salinities up to 13 ‰.

3. Symptoms of death are those of asthenia accompanied by a slight weight increase, averaging 4.3% in fish not being handled daily.

4. Serum chloride determinations showed that normal fish in either fresh or salt water maintain a uniform level of *ca.* 0.886 gm.% NaCl. Serum of hypophysectomized fish in salt water is not significantly lower (*ca.* 0.817 gm.%). Hypophysectomized fish dying in fresh water have only *ca.* 0.383 gm.%.

5. Replacement therapy with *Fundulus* pituitary brei enabled hypophysectomized fish to survive in fresh water. Pituitary extract from *Perca flavescens*, a strictly fresh water species, was partially effective, whereas extracts from glands of *Pollachius virens*, a marine species, had no beneficial influence.

6. Injections of ACTH, GH, TSH, thyroxin, DOCA, posterior lobe extract, and an ACTH-GH-TSH combination failed to enable the fish to survive in fresh water.

7. The data presented above appear to indicate that the pituitary of *F. heteroclitus* secretes an unknown factor(s) which regulates salt balance in that fish in fresh water. This factor is apparently lacking in the stenohaline marine species, *P. virens*, but may be present in the fresh water species, *P. flavescens*.

8. Hypophysectomy has no effect on the cytology of the chloride cells either in the active (sea water) or regressed (fresh water) condition.

9. Mucous cells were more abundant in the gills of normal fish when adapted to fresh water than when adapted to salt water. Hypophysectomy resulted in atrophy of the mucous cells in either medium and a decreased abundance in fish dying in fresh water. The abundance and condition of the mucous cells was restored to normal in hypophysectomized fish which were induced to survive in fresh water by injections of *Fundulus* pituitary brei.

10. The data suggest that the chloride cells are not concerned in the failure of hypophysectomized fish to survive in fresh water, but that atrophy of the mucous cells may be involved.

LITERATURE CITED

Abramowitz, A. A., 1937. The opercular approach to the pituitary. *Science*, **85**: 609.

Arvy, L., M. Fontaine and M. Gabe, 1954. Action des solutions salines hypertonique sur le système hypothalamo-hypophysaire, chez *Phoxinus laevis*, Agass., et chez *Anguilla anguilla*, L. *C. R. Soc. Biol.*, **148**: 1759–1761.

Arvy, L., M. Fontaine and M. Gabe, 1955. Actions des solutions saline hypertoniques sur le neurosecrétion hypothalamique chez l'anguille hypophysectomisée. *C. R. Soc. Biol.*, **149**: 225–228.

Baker, B. L., and G. D. Abrams, 1955. Growth hormone (somatotropin) and the glands of the digestive system. Internat. Symp. *The Hypophyseal Growth Hormone, Nature and Actions* (R. W. Smith, O. H. Gaebler, C. N. H. Long, Eds.) Blakiston, New York: 107–122.

Baldwin, E., 1937. Introduction to comparative biochemistry. Cambridge University Press, London. Pp. 17–33.

Bargmann, W., 1954. Die endokrine Funktion neurosekretorische Zellen bei Wirbeltieren. *Pubbl. Staz. Zool. Napoli*, **24**, *Suppl.*: 11–14.

Black, V. S., 1951. Some aspects of the physiology of fish. II. Osmotic regulations in teleost fishes. *Univ. Toronto Biol. Ser.*, **59**: 53–89.

BOUCHER-FIRLY, S., 1935. Recherches biochimique sur les téléostéens apodes (anguille, congre, murène). *Ann. Instit. Oceangr.* n.s., **15**: 108–113.

BOYD, E. M., AND M. DINGWALL, 1939. The effect of pituitary (posterior lobe) extract on the body water of fish and reptiles. *J. Physiol.*, **95**: 501–507.

BURGESS, W. W., A. M. HARVEY AND E. K. MARSHALL, JR., 1933. Site of the antidiuretic action of pituitary extract. *J. Pharmacol.*, **49**: 237–249.

CALLAMAND, O., 1943. L'anguille Européene (*Anguilla anguilla*, L.). Les bases physiologiques de sa migration. *Ann. Instit. Oceangr. Monaco*, **21**: 361–440.

CALLAMAND, O., M. FONTAINE, M. OLIVEREAU AND A. RAFFY, 1951. Hypophyse et osmorégulation chez les poissons. *Bull. Instit. Oceangr. Monaco No. 984:* 1–7.

CHAVIN, W., 1954. The role of the pituitary-adrenal mechanism in the reappearance of melanin and melanophores in the goldfish, *Carassius auratus*, L. Dissertation, New York University.

COPELAND, D. E., 1948. The cytological basis of chloride transfer in the gills of *Fundulus heteroclitus*. *J. Morph.* **82**: 201–227.

COPELAND, D. E., 1950. Adaptive behavior of the chloride cell in the gill of *Fundulus heteroclitus*. *J. Morph.*, **87**: 369–380.

COVENTRY, F. H., V. E. SHELFORD AND L. F. MILLER, 1935. The conditioning of a chloramine treated water supply for biological purposes. *Ecology*, **16**: 60–66.

D'ANGELO, S. A., 1955. Pituitary regulation of thyroid gland function. Brookhaven Symp., *The Thyroid:* 9–27.

DRILHON, A., 1942. Destruction de la moelle et adaptation aux changements de salinité d'un poisson homeoosmotique (carpe). *C. R. Acad. Sci. Paris*, **214**: 575–577.

FIRLY, S., 1932. Influence des variations de salinité sur la pression osmotique des civelles. *C. R. Soc. Biol.*, **110**: 247–248.

FONTAINE, M., 1953. Equilibre hydrominerale et quelques particularités de sa régulation chez les vertebres. *Arch. Sci. Physiol.*, **7**: C55–77.

FONTAINE, M., 1956. The hormonal control of water and salt-electrolyte metabolism in fish. *Mem. Soc. Endocrin.*, **5**. (In press).

FONTAINE, M., AND S. BOUCHER-FIRLY, 1934. Recherches sur la reserve alcaline du sang de poissons, ses variations au cours des changements de salinité. *Bull. Instit. Oceanogr. Monaco No. 646:* 1–12.

FONTAINE, M., AND O. CALLAMAND, 1948. Nouvelles recherches sur le determinisme physiologique de l'avalaison des poissons migrateurs amphibiotiques. *Bull. Mus. Nation. Hist. Nat. Paris* (2), **20**: 317–320.

FONTAINE, M., O. CALLAMAND AND M. OLIVEREAU, 1949. Hypophyse et euryhalinité chez l'anguille. *C. R. Acad. Sci. Paris*, **228**: 513:514.

FONTAINE, M., AND A. RAFFY, 1950. Le facteur hypophysaire de rétention d'eau chez les téléostéens. *C. R. Soc. Biol. Paris*, **144**: 6–7.

GARREY, W. E., 1905. The osmotic pressure of sea water and the blood of marine animals. *Biol. Bull.*, **8**: 257–270.

GAUNT, R., J. H. BIRNIE, W. R. ROSS, W. J. EVERSOLE AND C. M. OSBORN, 1951. The adrenal cortex in water metabolism. AAAS Symp. *Pituitary Adrenal Function.* (R. C. Christman, Ed.) AAAS, Washington: 102–108.

GETMAN, H. C., 1950. Adaptive changes in the chloride cells of *Anguilla rostrata*. *Biol. Bull.*, **99**: 439–445.

GLICK, D., 1949. Techniques of histo- and cytochemistry. Interscience, New York: 166 et seq.

GORBMAN, A., AND O. BERG, 1955. Thyroid function in the fishes *Fundulus heteroclitus, F. majalis*, and *F. diaphanus*. *Endocrin.*, **56**: 86–92.

GRAFFLIN, A. L., 1931. Urine flow and diuresis in marine teleosts. *Amer. J. Physiol.*, **97**: 602–610.

GRAFFLIN, A., 1935. Chloride and total osmotic pressure in the blood of marine teleosts. *Biol. Bull.*, **69**: 245–258.

GRAFFLIN, A. L., 1937. The problem of adaptation to fresh and salt water in the teleosts viewed from the standpoint of the structure of the renal tubules. *J. Cell. Comp. Physiol.*, **9**: 469–476.

HALL, B. A., I. E. GRAY AND S. LEPKOVSKY, 1926. The influence of asphyxiation on the blood constituents of marine fishes. *J. Biol. Chem.*, **67**: 549–554.

HAWK, P. B., B. L. OSER AND W. H. SUMMERSON, 1947. Practical physiological chemistry. Blakiston, Philadelphia: 574–575.

HENSCHEL, 1934–35. Wasseraushalt und Osmoregulation von Scholle und Flunder. *Wissenschaftliche Meeresuntersuchunges*, **22**: 91–121.

HOAR, W. S., 1951. Some aspects of the physiology of fish. I. Hormones in fish. *Univ. Toronto Biol. Ser.*, **59**: 1–51.

KALASHNIKOV, G. N., 1939. "The composition of the blood of fish" (In Russian). *Uchenye Zapiski MGU Hydrobiol.*, **33**: 65–103.

KALASHNIKOV, G. N., AND S. N. SKADOVSKII, 1948. "Ecological and physiological study of the sevriuga during the period of reproduction under natural and experimental conditions" (In Russian). *Zool. Zhurnal.*, **27**: 513–524.

KEYS, A., AND J. B. BATEMAN, 1932. Branchial responses to adrenalin and to pitressin in the eel. *Biol. Bull.*, **63**: 327–336.

KEYS, A., AND E. N. WILMER, 1932. "Chloride secreting cells" in the gills of fishes, with special reference to the common eel. *J. Physiol.*, **76**: 368–378.

KIRSHENBLATT, J. D., 1952. "The action of steroid hormones on female vy'un" (In Russian). *Doklady Akad. Nauk*, **83**: 629–632.

KOCH, H. J., AND M. J. HEUTS, 1942. Influence de l'hormone thyroidienne sur la régulation osmotique chez *Gasterosteus aculeatus*, L., form *gymnurus*, Cuv. *Ann. Soc. R. Zool. Belg.*, **73**: 165.

KROGH, A., 1939. Osmotic regulation in aquatic animals. Cambridge University Press, London.

LEVINSKY, N. G., AND W. H. SAWYER, 1952. Influence of the adenohypophysis on the frog water balance response. *Endocrin.*, **51**: 110–116.

LIU, C. K., 1942. Osmotic regulation and "chloride secreting cells" in the paradise fish—*Macropodus opercularis*. *Sinensia*, **13**: 15–20.

MATTHEWS, S. A., 1933. Color changes in *Fundulus* after hypophysectomy. *Biol. Bull.*, **64**: 315–320.

MARTINI, L., AND C. MORPURGO, 1955. Neurohumoral control of the release of adrenocorticotropic hormones. *Nature*, **175**: 1127–1128.

NISHIDA, H., 1953. The cytohistochemical observations on the gland cells of the branchial epidermis with the comparison of two types of Oncorynchus masou, landlocked and sea run forms. (In Japanese with English summary). *Sci. Rpts. Hokkaido Fish Hatchery*, **8**: 33-38.

PICKFORD, G. E., 1953. The hypophysectomized male killifish. *Bull. Bingham Oceanogr. Coll.*, **14** (2): 5-41.

PICKFORD, G. E., 1954a. The response of hypophysectomized male killifish to purified fish growth hormone, as compared with the response to purified beef growth hormone. *Endocrin.*, **55**: 274-287.

PICKFORD, G. E., 1954b. The response of hypophysectomized male killifish to prolonged treatment with small doses of thyrotropin. *Endocrin.*, **55**: 589–592.

PUSCHEL, J., 1928. Blutuntersuchungen bei einem Süsswasserteleostier (*Tinca vulgaris*, Cuv.). *Zeitschr. f. vergl. Physiol.*, **7**: 606–610.

SAWYER, W. H., 1956. The hormonal control of water and salt-electrolyte metabolism with special reference to the amphibia. *Mem. Soc. Endocrin.*, **5** (In press).

SMITH, D. C. W., 1956. The role of the endocrine organs in the salinity tolerance of trout. *Mem. Soc. Endocrin.*, **5** (In press).

STEVENS, C. E., AND C. P. LEBLOND, 1953. Renewal of the mucous cells in the gastric mucosa of the rat. *Anat. Rec.*, **115**: 231–243.

SUMNER, F. B., 1906. The physiological effects upon fishes of changes in density and salinity of water. *Bull. U. S. Bur. Fish.*, **25**: 53–108.

VARS, H. M., 1934. Blood studies on fish and turtles. *J. Biol. Chem.*, **105**: 135–137.

WHITE, H. L., 1955. Growth hormone and renal function. Internat. Symp., *The Hypophyseal Growth Hormone, Nature and Actions* (R. W. Smith, O. H. Gaebler, C. N. H. Long, Eds.). Blakiston, New York, 178–186.

WILHELMI, A. E., G. E. PICKFORD AND W. H. SAWYER, 1955. Initiation of the spawning reflex response in *Fundulus* by the administration of fish and mammalian neurohypophyseal preparations and synthetic oxytocin. *Endocrin.*, **57**: 243–252.

CYTO-EMBRYOLOGICAL STUDIES OF SEA URCHINS. III. ROLE OF THE SECONDARY MESENCHYME CELLS IN THE FORMATION OF THE PRIMITIVE GUT IN SEA URCHIN LARVAE

KATSUMA DAN [1] AND KAYO OKAZAKI [2]

Biology Department, Tokyo Metropolitan University, Tokyo, and Misaki Marine Biological Station, Miura-Shi, Japan

Analyses of the mechanism of gastrulation in the past have been based either on observations of the normal process or on studies of the effect of LiCl. When one proceeds along the first line, it is rather difficult to reach a decisive conclusion. When one selects the second approach, in addition to the failure of gastrulation, another, more profound, factor of changes in differentiating potency is introduced. Yet, since lithium treatment and exogastrulation are so consistently associated, there is a tendency to equate lithium treatment with the analysis of the mechanism of gastrulation. Obviously handling two variables simultaneously is a rather dangerous procedure. In a strict sense, therefore, before attacking the lithium effect, exogastrulation not accompanied by a change in differentiation capacity should be studied. This attitude is observed in the present paper.

Normal Process of Gastrulation

Concerning the mode of gastrulation in sea urchins, the views of such earlier workers as Herbst (1893, 1896), Morgan (1896), Schmidt (1904) can roughly be summarized as saying that the endodermal plate of the beginning gastrula gives rise only to the tip of the archenteron, while the blastular wall encircling the original endodermal plate invaginates, completing the formation of the gut. These investigators believed that the volume of the larva actually decreases during gastrulation because of the inward migration of so much of the blastular wall.

On the other hand, Hörstadius (1935, 1936, 1939) showed by a vital staining technique that the presumptive archenteron takes its origin from the veg_2-disk of the 64-cell stage. At the beginning of gastrulation, this entire mass invaginates and the gut is formed by the elongation of the mass. Hörstadius further asserted, in contrast to the conclusion of the previous workers, that the volume of the larva increases during gastrulation.

Although the process of gastrulation can be observed fairly clearly in the living larvae of any transparent forms, the observation can be further supplemented by following the development of individual larvae reared in a simple moist chamber as described below.

From a piece of tissue paper, the thickness of which is a little less than that of the larva, a square piece slightly smaller than a cover slip is cut out and a hole

[1, 2] The present work was partly supported by the Research Expenditure of the Ministry of Education for which the authors' thanks are due.

several millimeters in diameter is made at the center. After wetting the paper with sea water on a slide, a droplet of sea water containing one larva is placed at the center of the hole (but not touching the paper) and a cover glass is slowly laid down. The wet paper prevents evaporation and the droplet is the culture medium for the larva. If the entire preparation is put into a still larger moist chamber, or if distilled water is occasionally added to the paper (these methods are preferable to sealing the cover slip with vaseline), it is possible to rear the larva from the gastrula to the pluteus stage. In this small space, as the larva swims rather slowly,

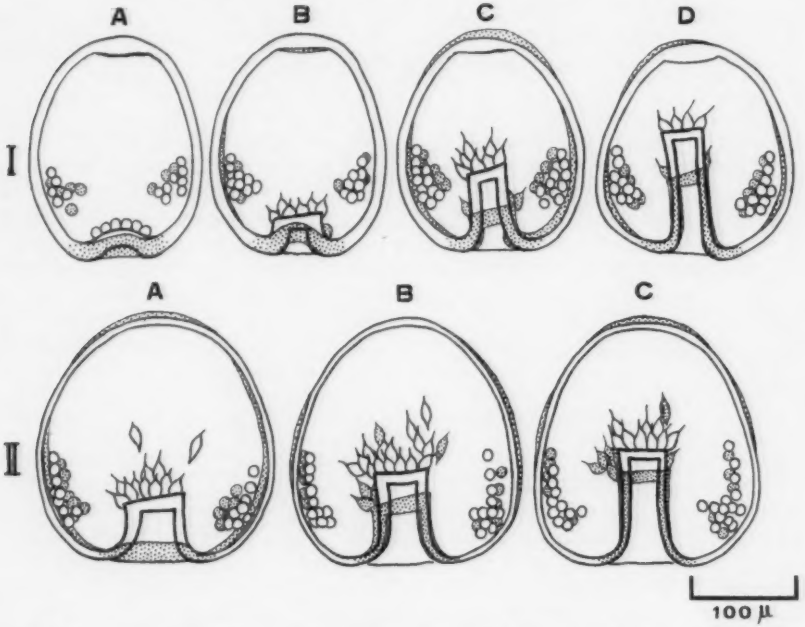

FIGURE 1. Superimposed contour-tracings of photographs of a single larva through the gastrulation process. Dotted drawings represent preceding stages and open drawings are succeeding stages. The upper row: *Clypeaster japonicus;* the lower row: *Mespilia globulus.*

photographs of various views of the larva can be made with a shutter speed of $\frac{1}{50}$ second. Further, in such droplets, although development is slowed down to some extent, no sign of morphological abnormalities was detected.

Figure 1 shows superimposed tracings of a series of photographs, taken at appropriate intervals, of the larvae of two kinds of sea urchin. As can be seen from the figure, throughout the period from the stage of endodermal plate formation to the completion of gastrulation, the contours of the larvae and the positions of the primary mesenchyme cells hardly change, while the archenterons increase their lengths considerably. Moreover, it will be noticed that as the archenteron elongates, the thickness of its wall decreases. The authors believe that this fact sup-

ports the conclusion of Hörstadius mentioned. The only discrepancy found is that the volume of the larva remains constant during the gastrulation process, and it is only after the completion of gastrulation that the volume increase takes place.

From both Hörstadius' results and these observations of the authors, the following facts seem to be certain:

1) All the material which will form the archenteron is first laid down as an endodermal plate preceding gastrulation.

2) At the beginning of gastrulation, the entire mass of this endodermal plate invaginates *in toto*.

3) The formation of the primitive gut is due to the stretching of the original endodermal plate, with no other elements included.

Formation of Pseudopodia by the Secondary Mesenchyme Cells

Soon after the onset of invagination of the endodermal plate, the secondary mesenchyme cells, differentiating at the tip of the archenteron, begin to send out fine pseudopodia.[3] These pseudopodia elongate toward the wall of the animal side of the larva, and by the time the archenteron attains a length ½–⅓ the diameter of the blastocoel, the ends of the pseudopodia are attached to the blastular wall either directly or by the mediation of a few cells arranged on top of the others.

After the establishment of the connection between the blastular wall and the pseudopodia, larvae are occasionally found in which the part of the blastular wall where the pseudopodia are attached is pulled in. In *Pseudocentrotus* larvae this effect is very conspicuous. In mid- or late gastrula stage, they frequently acquire two indentations, one on the animal side where the pseudopodia are anchored, and the other, the blastopore, on the vegetal side; thus the entire larvae somewhat simulate the shape of a mammalian red blood cell (see also Okazaki).

Moreover, if the shape of the tip of the archenteron is carefully observed, it will be seen that it seldom has a rounded contour, but it is of an edgy or squarish shape resembling a suspended fabric. In case a group of pseudopodia develops better on one side, the roof of the archenteron is lifted up higher toward the animal pole on that side. All these facts give the impression that the archenteric roof is being pulled toward the animal pole by the pseudopodia of the secondary mesenchyme cells. This interpretation is further consolidated by the following several experiments.

Injection of Oil Droplets into the Blastocoel

In order to find out whether mesenchymal pseudopodia are actually capable of pulling an object, the simplest way will be to introduce an easily movable object into the blastocoel. For this purpose, oil droplets were micro-injected into the blastocoels of newly hatched blastulae. When the primary mesenchyme cells appear, regardless of the position in the blastocoel of the oil droplets, they are caught by the primary mesenchyme cells. However, when the secondary mesenchyme cells are formed, their traction force is apparently stronger than that of the primary

[3] The time of appearance of the pseudopodia differs from one species to another. In *Mespilia*, in which they appear earliest, the pseudopodia are formed immediately after the initiation of invagination; in *Pseudocentrotus*, in which they appear late, the pseudopodia can be recognized clearly only after the archenteron reaches ⅓ of the distance across the blastocoel.

mesenchyme cells so that by the time the mid- or late gastrula stage is reached, the oil droplets are relayed over to the secondary mesenchyme cells and are drawn toward the archenteric tip. Some photographs are shown in Figure 2. It should be pointed out that since the injected oil drop is immediately surrounded by a protein film, the pseudopodia have no difficulty in getting hold of it.

Now having seen that the basic conditions are fulfilled, let us proceed to examine whether or not the pseudopodia are actually pulling up the archenteron.

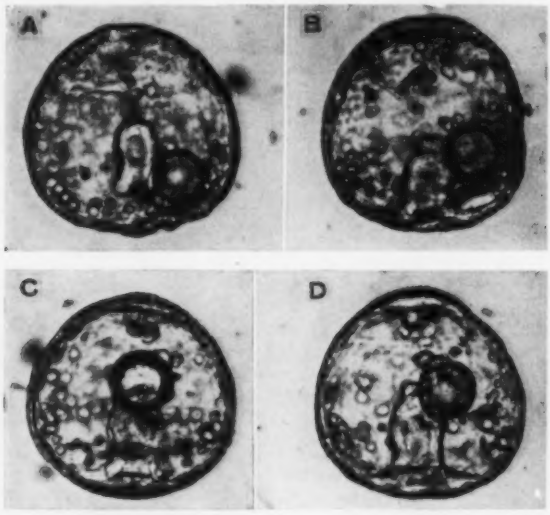

FIGURE 2. Microphotographs of *Clypeaster* larvae with oil drops injected into the blastocoels. A. Early stage. Oil droplet is caught by the pseudopodia of the primary mesenchyme cells. B. Intermediate stage. The oil is picked up by the pseudopodia of the secondary mesenchyme cells also. C, D. Later stages. As the pull by the secondary mesenchyme cells is stronger, the oil is removed from the primary mesenchyme cells and is drawn toward the archenteric tip.

EFFECTS OF EXPERIMENTAL INCREASE OF THE BLASTOCOELIC VOLUME

If the tip of the archenteron is actually being pulled up toward the animal pole by pseudopodia which have anchored themselves on the blastular wall, the pulling force will be increased if the blastocoelic volume is increased. This increased pull, in turn, would stretch the archenteron making it longer. Moore (1939, 1940) and Dan (1952) have reported that since the sea urchin blastular wall loses permeability to sucrose molecules after the tenth cleavage cycle (*i.e.*, at the time of hatching), if larvae cultured in a sucrose-containing medium are transferred to pure sea water after hatching, they become considerably inflated as the result of an abnormally high internal osmotic pressure developed by the sucrose molecules entrapped in the blastocoel.

Repetition of the sugar procedure on *Clypeaster japonicus* and *Mespilia globulus* gives not only a confirmatory result concerning the above expectation but it further

gives rise to exogastrulae in the proportion given in Table I. Figure 3 illustrates superimposed tracings of photographs of individual larvae of *Mespilia* during the process of inflation and of exogastrulation. As can be seen in the figure, during the few minutes following the transference from sucrose sea water to pure sea water when the larva is increasing in size, the pseudopodia are stretched and at the same time, the archenteron actually lengthens (Fig. 3, B, C). However, when the volume is further increased, the pseudopodia or chains of mesenchyme cells extending between the gastrular wall and the tip of the archenteron are broken and the cells round up. Under this circumstance, the archenteron begins to be pushed out of the blastocoel from its basal part (Fig. 3, F and Fig. 4).

Whenever the archenteric tip is wide and flat-topped, it was invariably found that some of the pseudopodia are anchored to the lateral wall instead of to the animal pole region so that they are pulling the archenteric tip sideways (Fig. 3, D).

TABLE I

Percentages of normal, entoexo- and exogastrulae obtained by inflation of the blastocoel by sugar treatment in Clypeaster japonicus and Mespilia globulus

Species	Stage of returning from sugar solution to sea water	10% sugar s.w.→ Natural s.w.			20% sugar s.w.→ Natural s.w.			30% sugar s.w.→ Natural s.w.		
		Invaginated form	Ento-exo-gastrula	Exo-gastrula	Invaginated form	Ento-exo-gastrula	Exo-gastrula	Invaginated form	Ento-exo-gastrula	Exo-gastrula
Clypeaster	Early gastrula	—	—	—	72.5%	15%	12.5%	92%	5.5%	2.5%
	Mid gastrula	—	—	—	65	14.5	20.5	25	21	54
Mespilia	Early gastrula	67%	5%	28%	43	12	45	—	—	—
	Mid gastrula	68	10	22	30	12	58	—	—	—
	Early gastrula	74	8	18	65	8	27	—	—	—
	Mid gastrula	28	32	40	18	24	58	—	—	—
	Late gastrula	69	26	5	62	25	13	—	—	—

At any rate, as long as the pseudopodial connection is intact, the archenteric tip takes an edgy contour like a suspended fabric, while if the connection is severed, the contour rounds off (Fig. 3, E, F).

Generally speaking, secondary mesenchyme cells are amoeboid. Therefore, they are continuously extending and retracting pseudopodia, forming new ones and abolishing old ones, and some cells move along other cells. When such pseudopodia are broken as the result of blastocoelic expansion, although the secondary mesenchyme cells round up for a while, sooner or later they begin to send out new pseudopodia. In a larva in which only the basal part of the archenteron is evaginating, if a new pseudopodial connection is established in the normal position, the archenteric tip begins to be pulled back toward the animal pole once more. As a matter of fact, even in larvae in which the entire archenteron has been everted, the archenteric tip can still resume the inward movement when a connection is successfully established. Because of these secondary connections, various degrees of entoexogastrulae result in an experimental batch. Some of these are depicted in Figure 4, A–F.

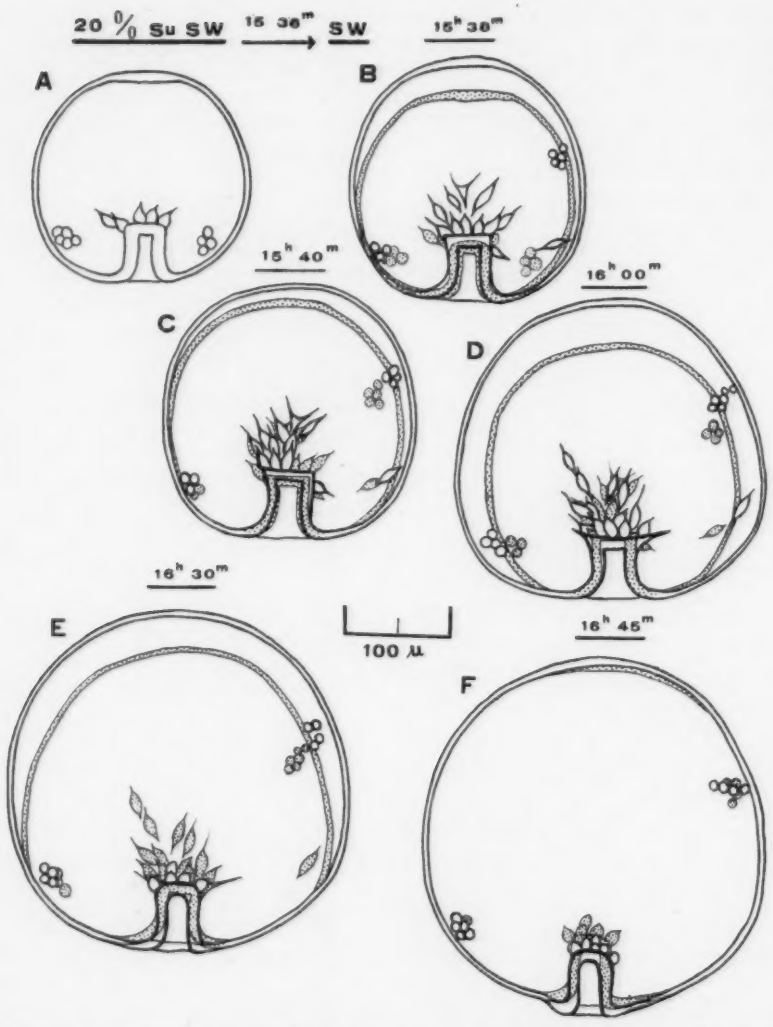

FIGURE 3. Effects of inflation of the blastocoel on the pseudopodia of the secondary mesenchyme cells and on the archenteron of *Mespilia globulus*. A. A larva in a sugar-containing sea water before transfer. B, C. Transfer to pure sea water. As a result of blastocoelic expansion, the pseudopodia are stretched, in turn pulling the archenteron longer. D. Flat-topped archenteron indicates that the pseudopodial connection to the animal pole has been severed while the connection to the side wall is still preserved. E. Rounded tip of the archenteron indicates the severance of the pseudopodial connection and the archenteron is receding. F. The secondary mesenchyme cells have also rounded up and the gut is evaginating.

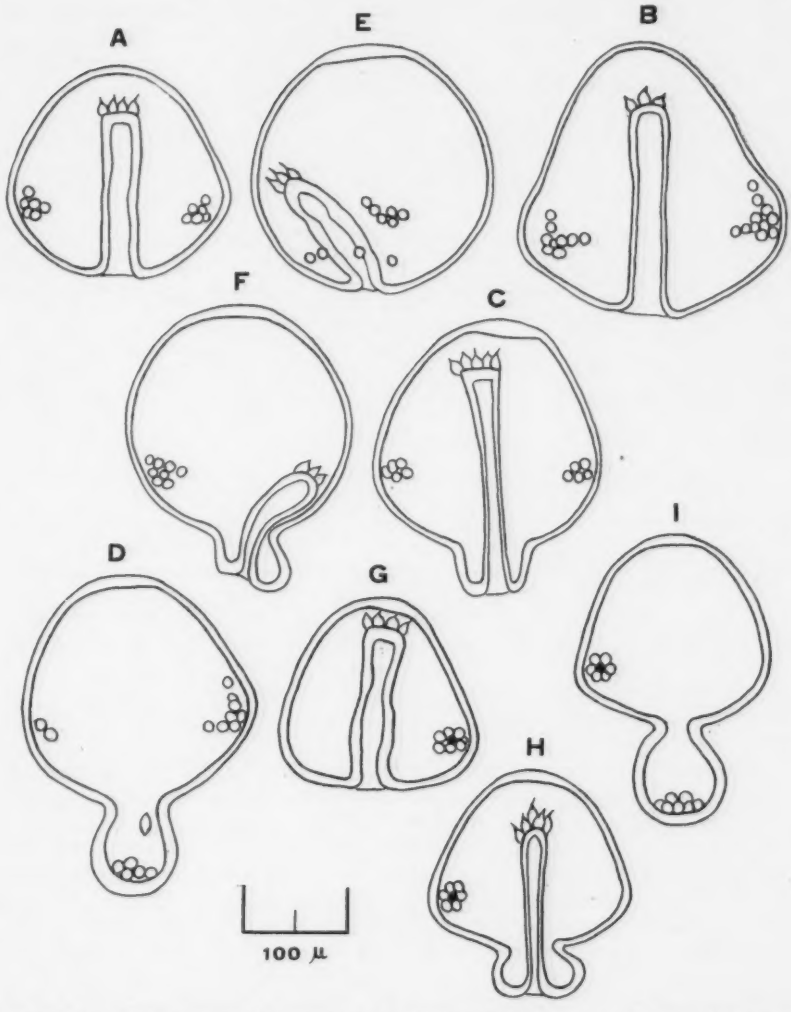

FIGURE 4. Various types of abnormality in *Clypeaster japonicus*. A-F: Transferred from sugar to pure sea water at early gastrula stage. A. Control in sugar-sea water. B. Inflated larva which has successfully invaginated. Note the height of the archenteron and the thinness of its wall. C. An entoexogastrula. The wall of the gut is thinned out considerably. D. An inflated larva with a completely evaginated gut. The wall of the gut is very thick. E. An inflated larva in which the pseudopodia have attached themselves to the side wall. F. The same as E with the gut going astray. G-I: Exposed to Ca-low condition ($\frac{1}{10}$ that of sea water) from the gastrula stage onward. G. Control for Ca-low series. H. An entoexogastrula caused by the Ca-low treatment. I. Exogastrula in $\frac{1}{10}$ Ca.

From Figure 4 it becomes evident that the completely everted archenteron of an exogastrula (D) is shorter and its wall is thicker than the control (A), while the invaginated part of the gut of an entoexogastrula is highly stretched and its wall is extremely thin (C). This result is to be expected in the latter type, since a part of the gut having been everted, the remaining fraction has to supply all the material to be pulled out to reach the animal pole. Comparison of Figures 4 A and 4 B shows that even among successfully invaginated larvae, a proportionality is evident between the size of the blastocoel and the length of the gut or the thinness of the archenteric wall.

From the above observations, the following points can be inferred. When the blastocoel is abruptly inflated by a transfer from a sucrose medium to sea water, the pseudopodia of the mesenchyme cells tend to break and the primitive gut retreats. But judging from cases of entoexogastrulae, if the pseudopodial connection is re-established, its mild but continued pull can eventually pull out the primitive gut so that it becomes longer than normal (C). The short, bag-like shape of the completely everted archenteron of an exogastrula (D) must be produced under the

TABLE II

Percentages of normal, entoexo- and exogastrulae caused by Ca-low treatment in Clypeaster

S.W.....1/10 Ca

Stage of transfer	Experiment 1			Experiment 2		
	Invaginated	Entoexogastrula	Exogastrula	Invaginated	Entoexogastrula	Exogastrula
Early gastrula	31%	16%	53%	18%	0%	82%
Mid gastrula	39	34	27	74	7	19
Late gastrula	87	10	3	96	2	2
Post gastrula	97	3	0	100	0	0

complete absence of such an extrinsic traction. By the time the normal larva reaches the pyramid stage, however, the everted gut of the exogastrula grows in length to a limited extent and eventually exhibits a perfect tripartite formation. This fact indicates that even in the everted gut, tissue differentiation can take place perfectly, although such a gut never assumes the cylindrical shape typical of normal gastrulae of an advanced stage. This, in turn, indicates that the typical cylindrical shape and squarish tip of the normal archenteron is brought about by the pulling force of the pseudopodia. On the other hand, an abnormally stretched archenteron of an entoexogastrula often breaks in the thinnest part, and the upper portion goes to the animal pole while the basal portion evaginates completely. The result is a larva which has a stomodaeum and a pharynx at the normal position but with the stomach and intestine everted. This is obviously a case in which the primitive gut has yielded to the pulling force.

Among larvae which have been subjected to the sucrose treatment, such forms as those shown in Figure 4 E and 4 F are also encountered, in which the tip of the archenteron has failed to reach the stomodaeum although the gut as a whole remained inside the blastocoel. The cause for this lies in the fact that when new pseudopodia were formed, they attached themselves to the side wall instead of to

the wall of the animal pole and as a consequence, the archenteric tip is led to a wrong destination.

Suppression of Pseudopodial Formation of the Mesenchyme Cells

From what has been said, it can be expected that exogastrulae will also result if the pseudopodia are made to retract instead of being broken. In order to cause already formed pseudopodia to disappear, there are three methods available.

1) In *Clypeaster,* if the larvae are transferred to Ca-low sea water ($\frac{1}{10}$ Ca concentration of sea water) at the gastrula stage when pseudopodia are being formed by the secondary mesenchyme cells, the pseudopodia disappear in some of the larvae and exo- and entoexogastrulae similar to those obtained by the inflation technique are produced (Fig. 4, G–I). The frequency of their occurrence is given in Table II. Closer tracing of the development of a single larva reveals that the suppressing effect of the Ca-low medium works rather slowly and for a few hours pseudopodia can be seen, during which time the archenteron keeps on elongating little by little. But as soon as the pseudopodia disappear completely, the archenteron begins to retreat. The correlation between the two is quite definite, as will be seen in Figure 5, I.

In the Ca-low batch, various gradations of suppression are seen: complete suppression giving exogastrulae (Fig. 5, I), resumption of pseudopodia formation resulting in entoexogastrulae (II), formation of very slender and weak pseudopodia enough to prevent exogastrulation but failing to bring the archenteric tip to the stomodaeum (III), and finally quasi-normal larvae (Fig. 5, IV). In the III class of larvae, the secondary mesenchyme cells form an unpaired coelom at the tip of the primitive gut, thus preventing the union between the archenteron and the stomodaeum.

2) In *Mespilia,* if the gastrulae are exposed to pancreatin (0.5 to 0.125% in sea water), the pseudopodia are abolished in a short time and exogastrulae are formed (Fig. 6). The percentages of entoexo- and exogastrulae are given in Table III. Besides the suppression of the pseudopodia, the pancreatin has secondary effects such as decreasing the larval volume and causing the blastular wall to wrinkle. This latter effect is presumably due to the action of pancreatin on the intercellular matrix and on the hyaline layer. However, concerning the hyaline layer, Moore (1952) has shown that this layer does not play an essential role in the gastrulation process. Because of these secondary effects, it is impossible to rear the larvae in the pancreatin solution indefinitely. However, if they are returned to sea water in time, larvae similar to those shown in Figures 4 and 5 for *Clypeaster* can develop.

3) Among *Mespilia* larvae kept in normal sea water, it sometimes happens that gastrulation proceeds seemingly normally during the early phase, but later spontaneously deviates from the normal course and leads to exo- or entoexogastrula formation. Even in such spontaneous cases, coincidence in the time of pseudopodial disappearance and evagination is always recognized.

Total Inhibition of Pseudopodia Formation

This can be achieved in *Pseudocentrotus* larvae by rearing them in a Ca-low medium ($\frac{1}{20}$ to $\frac{1}{10}$ normal Ca level) from an early stage. In this case, no sign

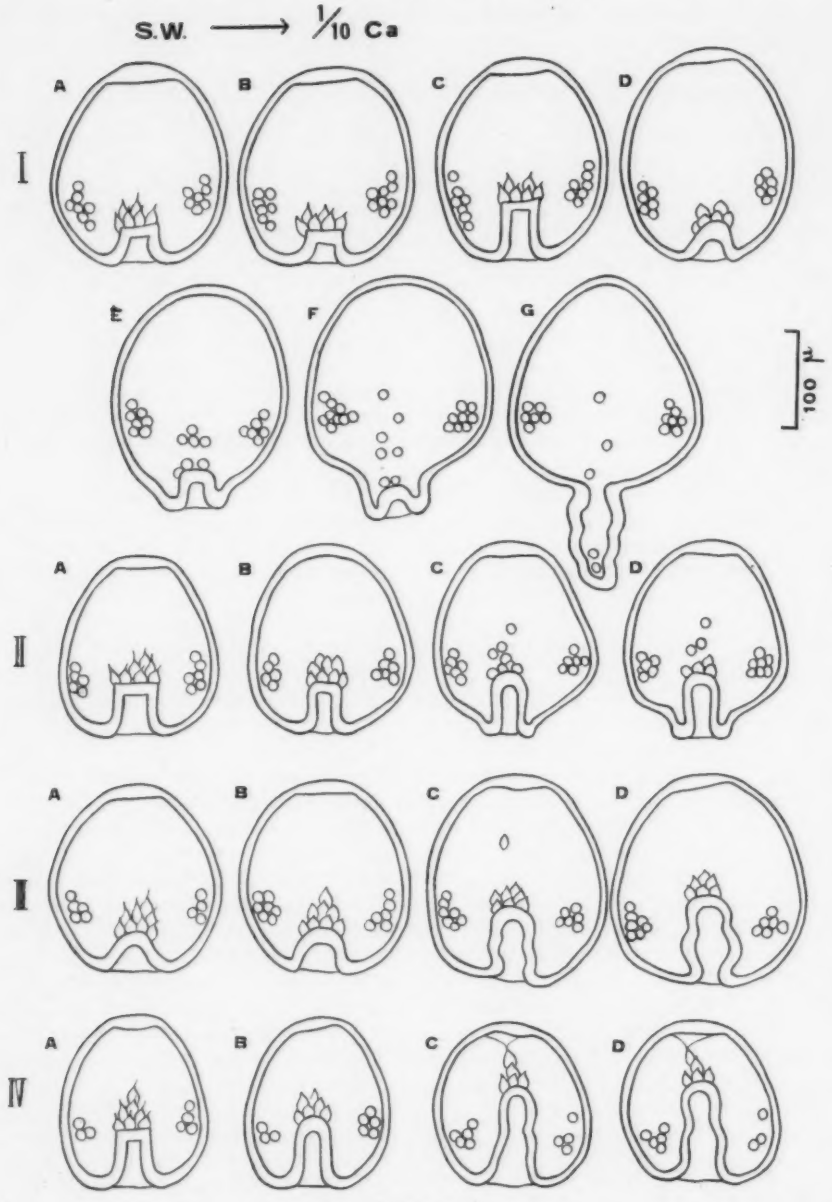

FIGURE 5.

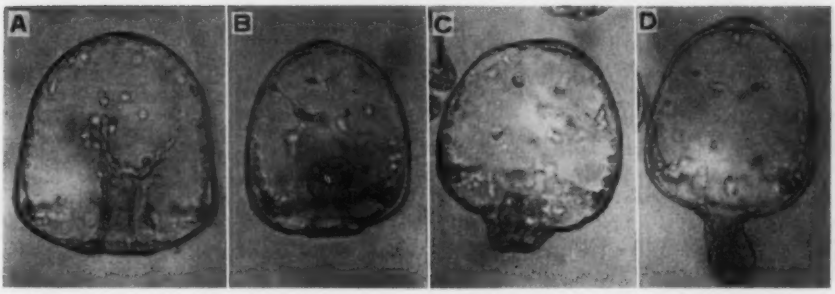

FIGURE 6. Course of exogastrula formation by pancreatin-sea water in *Mespilia globulus*.

of pseudopodia can be found and the archenteron extends ⅓ to ½ of the way across the blastocoel and stops there. By the time normal larvae in sea water have completed gastrulation, the archenterons of the larvae in the Ca-low medium gradually begin to retreat and they finally form exogastrulae. (A more detailed account of this process will be given in another paper by Okazaki.)

TABLE III

Percentages of normal, entoexo- and exogastrulae caused by pancreatin treatment in Mespilia

Stage of transfer	Pancreatin concentration								
	0.5%			0.25%			0.125%		
	Invaginated	Entoexogastrula	Exogastrula	Invaginated	Entoexogastrula	Exogastrula	Invaginated	Entoexogastrula	Exogastrula
Early gastrula	3%	15%	82%	12%	10%	78%	37%	7%	56%
Mid gastrula	12	18	70	24	26	50	34	6	60
Late gastrula	40	14	46	13	19	68	55	10	35
Post gastrula	88	7	5	91	5	4	89	2	9

DISCUSSION

The experimental results reported in this paper have shown that the pseudopodia of the secondary mesenchyme cells play an indispensable role in the formation of the primitive gut of sea urchin larvae. However, it is admitted that the pulling of the mesenchymal pseudopodia alone is not sufficient to account for all the power required to make a young endodermal plate change into the cylindrical gut of a late gastrula. For one thing, the endodermal plate begins to invaginate at the very be-

FIGURE 5. Suppression of the pseudopodia of the secondary mesenchyme cells of *Clypeaster japonicus* by 1/10 Ca that of sea water. The first figure (A) for each series is a larva immediately before transfer to the Ca-low medium. I. Complete suppression and resulting exogastrula formation. II. Partial recovery of the pseudopodia leading to an entoexogastrula. III. Feeble pseudopodia just strong enough to keep the archenteron from eversion but failing to draw it to the stomodaeal position. Such a larva acquires an unpaired coelom intervening between the stomodaeum and the archenteron. IV. A seemingly perfect larva in 1/10 Ca.

ginning of gastrulation, before the secondary mesenchyme cells start to form pseudopodia. Consequently, the pseudopodia cannot in any way determine whether the endodermal plate invaginates or evaginates. For another thing, such an endodermal plate can elongate by its own power without the cooperation of the pseudopodia. Probably it is justifiable to think that the maximal limit of this autonomous elongation is shown by the length of completely everted gut of an exogastrula.

Then what is the factor which causes this autonomous elongation of the endodermal plate? As has been realized by many previous workers, the only clue available at present is the change in shape of the cells composing the endodermal plate. While they are in the endodermal plate, they are tall and cylindrical. But as the invagination proceeds, they flatten out laterally. This change takes place even when the endodermal plate evaginates from the beginning. However, a point of importance is the fact that this flattening normally occurs in such a direction that it can be magnified by the pull by the pseudopodia.

Thus, the authors view the situation as follows: within the process of gut formation, three factors must be operating; (1) a factor responsible for the initiation of the invagination of the endodermal plate, (2) a process bringing about the form change of the constituent cells of the endodermal plate and (3) the traction exerted by the pseudopodia. During the early gastrula stage, the first two are acting while in the late gastrula stage, the second and third must be cooperating.

Concerning the first factor, which initiates invagination in the endodermal plate, various views have been expressed. Long ago Rhumbler (1902) attempted an analysis of the process on the premise that three factors—(a) pressure within the blastular wall resulting from increase in cell number, (b) pressure by a confining membrane and (c) absorption of blastocoelic fluid—might be playing some role. But after showing that none of these was involved, he tried to attribute the cause of inpocketing to surface tension changes in the vegetal cells. Morgan (1927) and a few others considered that it might be due to the pressure of dividing cells acting in a plane passing through the apical and basal poles of the embryo. Agrell's recently published opinion (1954) hints at a similar possibility. However, Morgan's idea was negated by Moore (1939). Moore himself contends that the invaginating force resides within the endodermal plate itself and this force decreases along the radial gradient with a maximum at the center. He attempted to calculate this force, using the results of his sugar experiments (1941).

Since then, Lewis (1947) has suggested for amphibian invagination that the superficial gel layer might be playing a role. Moore (1952) went on to test the validity of Lewis' idea in sea urchins and concluded that the hyaline layer of sea urchins is not essential for gastrulation (see also Dan, 1952). Gustafson and Lenique (1952) try to explain it by an increased pressure within the wall caused by a structural protein which is derived from mitochondria.

With respect to the form change of the cells making up the endodermal plate, Gustafson suggests the same explanation, that is, stretch by a structural protein. However, it must not be forgotten that besides simply elongating, such a gut is also able to self-differentiate.

Finally, the process of development of a functional digestive tract in sea urchin larvae requires a fusion between the tip of the archenteron and the gastrular wall at the stomodeal position. The authors believe that the observations presented in

this paper show conclusively that this is accomplished by the directing and pulling activity of the secondary mesenchyme cells, before they begin their main task of forming the coelom.

However, the authors do not hesitate to admit that the elucidation of the mechanism of the gastrulation process is still remote from a solution. What causes the formation of the endodermal plate to begin with? What factor initiates its invagination? What factors are involved in the autonomous elongation and self-differentiation of the primitive gut? How do the secondary mesenchyme cells know the site of the stomodaeum? All these important questions are left unanswered.

Summary

1. The first half of the gastrulation process in sea urchins begins with an *in toto* invagination of the endodermal plate, followed by its stretching.

2. In the second half of the process, pseudopodia are sent out by the secondary mesenchyme cells toward the animal pole. These pseudopodia attach to the blastular wall and pull the archenteron up toward that pole.

3. The pulling capacity of the pseudopodia is shown by the displacement toward the secondary mesenchyme cells of oil droplets injected in the blastocoel.

4. When the pseudopodial connection is artificially severed by one of the following methods, exogastrulae result:

a) Blastocoelic expansion after sucrose treatment (*Mespilia globulus, Clypeaster japonicus*).

b) Ca-low treatment (*Clypeaster, Pseudocentrotus depressus*).

c) Pancreatin treatment (*Mespilia*).

5. Abnormal invagination in various degrees brought about by the above treatments can be accounted for by differences in their effects on the pseudopodia formation.

6. Pseudopodia of the secondary mesenchyme cells are indispensable in the latter half of the gastrulation process in order to produce normal embryos.

LITERATURE CITED

Agrell, I., 1954. A mitotic gradient in the sea urchin embryo during gastrulation. *Arch. f. Zool.*, **6**: 213–217.

Dan, K., 1952. Cyto-embryological studies of sea urchins. II. Blastula stage. *Biol. Bull.*, **102**: 74–89.

Gustafson, T., and P. Lenique, 1952. Studies on mitochondria in the developing sea urchin egg. *Exp. Cell Res.*, **3**: 251–274.

Herbst, C., 1893. Experimentelle Untersuchungen über den Einfluss der veränderten chemischen Zusammensetzung des umgebenden Mediums auf die Entwicklung der Tiere II. Weiteres über die morphologische Wirkung der Lithiumsalze und ihre theoretische Bedeutung. *Mitt. Zool. Staz. Neapel*, **11**: 136–220.

Herbst, C., 1896. Experimentelle Untersuchungen über den Einfluss der veränderten chemischen Zusammensetzung des umgebenden Mediums auf die Entwicklung der Tiere. V. Über die Unterdruckung von Entwickelungsprozessen. *Arch. f. Entw.*, **2**: 482–497.

Hörstadius, S., 1935. Über die Determination im Verlaufe der Eiachse bei Seeigeln. *Pubbl. Staz. Zool. Napoli*, **14**: 251–429.

Hörstadius, S., 1936. Weitere Studien über die Determination im Verlaufe der Eiachse bei Seeigeln. *Arch. f. Entw.* **135**: 40–68.

Hörstadius, S., 1939. The mechanics of sea urchin development by operative methods. *Biol. Rev.*, **14**: 132–179.

LEWIS, W. H., 1947. Mechanics of invagination. *Anat. Rec.*, **97**: 139–155.

MOORE, A. R., AND A. S. BURT, 1939. On the locus and nature of the forces causing gastrulation in the embryo of *Dendraster excentricus*. *J. Exp. Zool.*, **82**: 159–168.

MOORE, A. R., 1940. Osmotic and structural properties of the blastular wall in *Dendraster excentricus*. *J. Exp. Zool.*, **84**: 73–79.

MOORE, A. R., 1941. On the mechanics of gastrulation in *Dendraster excentricus*. *J. Exp. Zool.*, **87**: 101–111.

MOORE, A. R., 1952. The process of gastrulation in trypsin embryos of *Dendraster excentricus*. *J. Exp. Zool.*, **119**: 37–46.

MORGAN, T. H., 1896. Experimental studies of the blastula and gastrula stages of Echinus. *Arch. f. Entw.*, **2**: 257–280.

MORGAN, T. H., 1927. Experimental embryology. Columbia University Press, New York.

RHUMBLER, L., 1902. Zur Mechanik des Gastrulationsvorgänges insbesondere der Invagination. *Arch. f. Entw.*, **14**: 401-476.

SCHMIDT, H., 1904. Zur Kenntnis der Larvenentwicklung von *Echinus microtuberculatus*. *Verh. phys. -med. Ges. Wurzburg*, **36**: 297.

GROWTH RATES IN LATITUDINALLY AND VERTICALLY SEPARATED POPULATIONS OF MYTILUS CALIFORNIANUS [1]

PAUL A. DEHNEL [2]

Department of Zoology, University of California, Los Angeles 24, California

Many marine poikilothermal species which encounter a relatively wide temperature difference over a latitudinal or vertical distribution, or over the annual cycle of seasons, demonstrate a compensatory response in various physiological activities. Rao (1953) has shown that the pumping rate of *Mytilus californianus* was higher for northern latitude animals at any given temperature when this activity was compared with southern ones. Segal (1955) compared heart rate of high and low intertidal populations of *Acmaea limatula* and found that low intertidal animals behaved as if they were cold-adapted relative to high intertidal animals. This has also been shown for *Mytilus californianus* for rate of water propulsion (Segal, Rao and James, 1953). These studies suggest a homeostatic tendency in many rate functions. The literature relevant to the problem of acclimation is extensive and has been reviewed recently (see Bullock, 1955 and Prosser, 1955).

With regard to rate of growth, usually, it is assumed that marine poikilotherms which inhabit high latitude seas grow more slowly, grow to a larger size and have a greater longevity, when compared with individuals of the same or closely related species from warmer seas. Such an assumption suggests that rate of growth is a simple function of temperature. Most studies reported in the literature indicate temperature-dependence, and do not, as in the case in metabolic studies, suggest compensation for temperature difference. However, Dehnel (1955) has compared rates of growth of larvae of several species of intertidal gastropods from southeastern Alaska and southern California. These results have shown that northern larvae grew more rapidly at all experimental temperatures, when compared with southern larvae of the same species. A discussion of some of the literature concerning rates of growth and latitude will be found elsewhere (Dehnel, 1955).

The purpose of this study is two-fold: to determine whether rates of growth in populations of the California sea mussel, *Mytilus californianus*, from southeastern Alaska and southern California are adapted in a compensatory direction relative to the temperature difference; to determine the effect of tidal height upon growth rate in this species. Few studies have concerned the latter aspect of any species. Coulthard (1929) compared growth in vertically separated populations of *Mytilus edulis*. These results showed rates of growth to be greater in completely submerged populations. Moore (1934) compared growth in populations of *Balanus*

[1] Aided in part by a grant from the Arctic Institute of North America under contractual arrangements with the Office of Naval Research. The author wishes to express his gratitude to Dr. Theodore H. Bullock, for many helpful suggestions and for critical reading of the manuscript.

[2] Present address: Department of Zoology, University of British Columbia, Vancouver 8, Canada.

balanoides located at different tidal levels, and found that growth was greatest at higher tidal levels except during the first year. Hatton (1938) studied rates of growth in high and low intertidal populations of *Balanus balanoides, Chthamalus stellatus* and *Patella vulgata*. This work revealed that young barnacles located at lower levels grew faster, but older animals grew more rapidly at higher intertidal levels. Reciprocal transplants of high and low intertidal animals showed that lower levels were still unfavorable for growth. Results obtained for *Patella vulgata* indicated that low level populations of this species grew more rapidly when compared with high level ones.

Rates of growth in adults of *Mytilus californianus* were studied by marking and measuring individuals *in situ* in the intertidal and allowing them to remain in the beds, in this manner obtaining natural rates of growth. The period of the study was chosen to be only a limited part of a year, during the growing season.

Material and Methods

Two geographic localities were chosen for this study: Big Rock, Los Angeles County, California (latitude 34° 00′ N., longitude 118° 30′ W.), and Mount Edgecumbe, Alaska (latitude 57° 03′ N., longitude 135° 20′ W.). Time spent at the northern locality extended from June to September, 1952, and July to August, 1953. The mean difference in surface sea water temperature between the two localities during the summer months was about 7° C.

It was regarded as important that rates of size increase in this species be compared at a season when growth was the most rapid. Coe and Fox (1942) showed that mussels from La Jolla, California grew most rapidly during April and May. The temperature of the sea water at that time was about 16° C. Water temperatures around Big Rock, California were not significantly different. In addition to the spring months thus selected for this study, the southern California mussels were measured in November, following the return from the northern locality. In southeastern Alaska it is assumed that comparable conditions for growth occur somewhat later, although no new data justifying this were obtained. In order to show the effect of vertical position upon growth, means of the absolute increase per thirty days in the shell dimensions were plotted as a function of intertidal height (Fig. 4). This plot is presented as it does not seem justified to determine a mean instantaneous relative growth rate value (to be discussed later) for the entire growth period, and the data obtained by Coe and Fox (1942) were desired for comparison.

Big Rock mussels were initially marked and measured April 13, 1952. A second series of measurements was made May 25, 1952 and the last series, November 1, 1952. Mussels were measured in 1954 on the following dates: April 24, May 22, July 1 and July 30. Mussels at Mount Edgecumbe, Alaska were measured on the following dates: June 26, July 24 and September 4, 1952.

Measurements of the shell were used. Individuals were marked by filing an identification mark on the shell and were then allowed to remain in the mussel beds for the period of study. Particular effort was made not to disturb each mussel any more than was necessary. A firm attachment by the byssal threads was confirmed, or the animal was discarded initially. Dial calipers were used for the measurements, which had an accuracy of ± 0.1 mm. The three shell measurements used were the greatest shell length, width and height, except that length was not ob-

tained for the 1952 sample from southern California. About two hundred individuals were measured in Alaska, and a like number in California.

To compare rates of growth of an attached species from different localities, it is necessary to locate groups of animals that occupy approximately comparable intertidal positions, with respect to wave action, degree of tidal exposure, compactness of the beds and size of animals that compose the bed. These parameters were difficult to measure, and assurance of ecologically similar conditions could be made only in a subjective way. The mussel beds at Mount Edgecumbe, Alaska and Big Rock, California were exposed directly to the open surf; the size of the animals was about the same (southern populations were somewhat larger); the mussel beds at the two localities were about the same in numbers of individuals per given area. On the causeway at Mount Edgecumbe, which consisted of a constructed road that served to connect several islands, and projected into the open ocean, the ecological distribution of *Mytilus californianus* was somewhat different from that observed at Big Rock. The intertidal zone is essentially vertical, and the gently sloping rocky coast so characteristic of southern California is lacking. Hence, it was necessary to attempt to locate mussel beds on a nearly vertical substratum that would be comparable, intertidally, to mussel beds in the south that had much greater horizontal distribution. The mussels in southern California were located at the 3.0 ft. ($\pm$ 0.5 ft.) and 1.0 ft. ($\pm$ 0.5 ft.) tide level and those in Alaska, at the 5.5 ft. ($\pm$ 0.5 ft.) level (zero datum level is mean lower low water). The lower figures demark the lower level of vertical distribution of this species at the respective localities. It should be noted that at both localities populations of *Mytilus californianus* were distributed vertically in the intertidal to well-delineated levels, below and above which the populations were essentially non-existent. This does not take into account subtidal populations recently located on the southern California coast (Berry, 1953; Rao, 1953). Values given elsewhere in the text for hours and percentage exposure per week at these levels were calculated from Ricketts and Calvin (1952, Fig. 112).

Under field conditions the problem of recovery of marked animals was serious. Within one month there may occur a great deal of settling of barnacle larvae, young mussels and seaweed, thus obscuring marked animals. Some individuals moved along or within the bed. This movement may be over the surface of the bed, or, as in many cases, mussels moved vertically from the surface of the bed several inches down to the rock substratum. One month following a series of measurements, about 60% recovery was realized and two months later, approximately 40% of the remainder. At lower intertidal levels in southern California about 80% returns were obtained over the period of four months. It is assumed that the losses were non-selective with respect to the size of the animals.

Method of Analysis

As yet no adequate formula has been propounded to describe all phases of growth in any animal (Weymouth, McMillan and Rich, 1931; Huxley, 1932; Brody, 1945). For this material the most suitable expression is the instantaneous (true) relative growth rate $(k) = \frac{dL/dt}{L}$. A discussion of the significance and justification for using this method will be found elsewhere (Dehnel, 1955). The

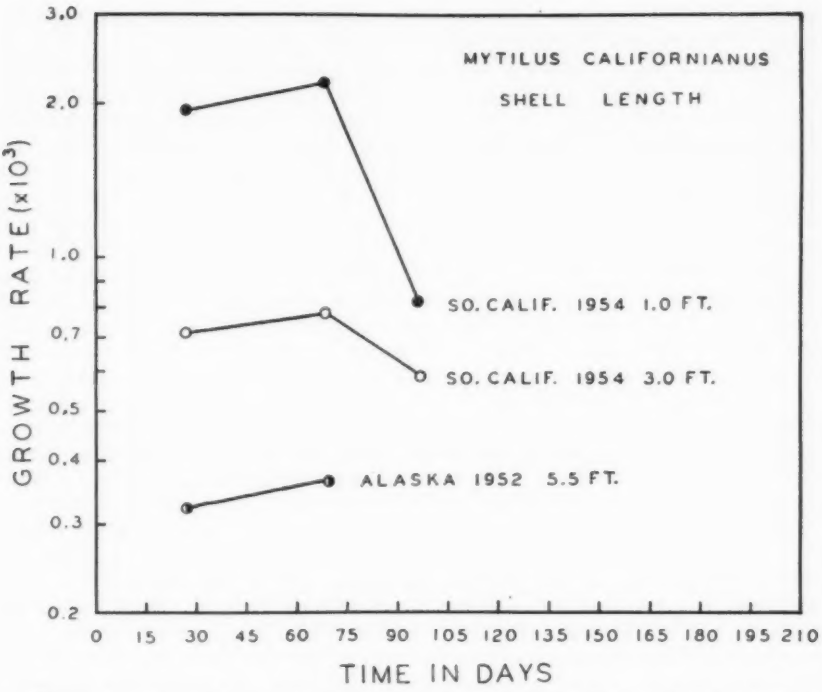

FIGURE 1. A comparison of the rate of growth in length of shell in populations of *Mytilus californianus* from Mount Edgecumbe, Alaska at one intertidal level, and Big Rock, California at two intertidal levels. Each point represents the mean k value for a sample of fifty or more individuals. The initial value is plotted on the day at which the second measurement was made.

instantaneous relative growth rates of the length, width and height of the shell were plotted as a function of time (Figs. 1, 2, 3). The initial value is plotted on the day at which the second measurement was made.

The means of the initial length, width and height of the shell are about 25% greater for the southern California mussels sampled than for northern ones. Presumably, similar size in latitudinally separated populations of a given species indicates different chronological ages, the northern ones being older (Wimpenny, 1941). Hence, samples of the northern and southern populations under consideration should be approximately the same age. A small initial size was chosen, as growth rates are more rapid in young animals.

RESULTS

Growth rate in latitudinally separated populations

When the instantaneous relative growth rate of the length of the shell is plotted as a function of time (days), it is seen that the rate of linear size increase of this

dimension is greater in the population from southern California at the 3.0 ft. level (Fig. 1) than in the Alaskan one (5.5 ft. level). The rate of the northern sample is about 45% of the southern one, initially, but the slopes of the two curves are nearly parallel. The mean absolute increase per thirty days for the southern California sample over the period from April 24 to July 30, 1954 was 0.96 mm.; for the smaller mussels from Alaska (June 26 to September 4, 1952), the increase was 0.33 mm. In the middle part of the summer, the rate for the southern sample de-

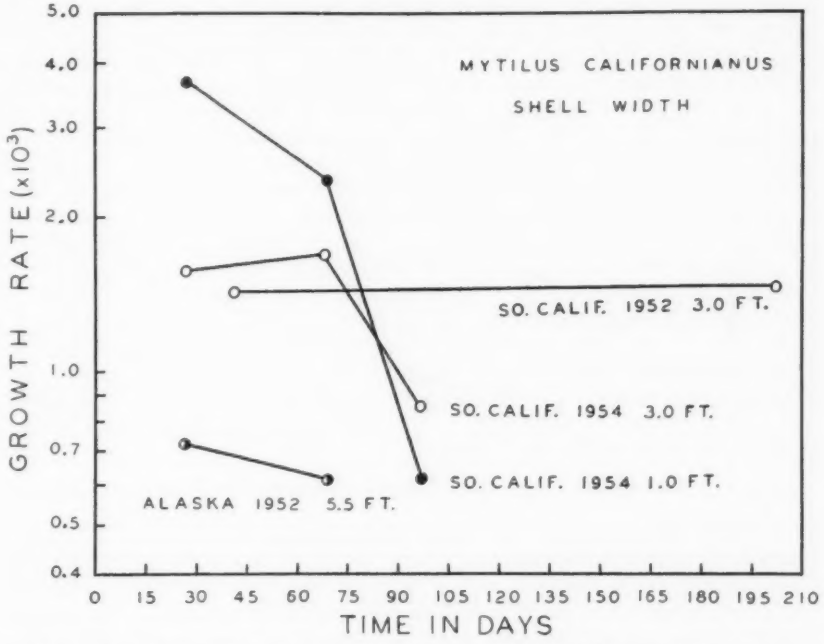

FIGURE 2. A comparison of the rate of growth in width of shell in populations of *Mytilus californianus* from Mount Edgecumbe, Alaska at one intertidal level, and Big Rock, California at two intertidal levels. Each point represents the mean k value for a sample of fifty or more individuals. The initial value is plotted on the day at which the second measurement was made.

creases, but is still growing at a rate greater than the one from Alaska, as seen from an extrapolation of the northern curve.

Also, the rate of linear increase in shell width is greater in the population from the 3.0 ft. level from southern California when compared with the population at the 5.5 ft. level from Alaska (Fig. 2). The rate of the northern sample, again, is about 45% of the southern one. The initial rates of the two samples from southern California are very similar. The 1952 sample shows no decline in rate due to the lack of intermediate points. For the years 1952 and 1954 the mean absolute increase per thirty days was 0.90 mm. and 0.81 mm., respectively. The mussels from Alaska showed a 0.30 mm. increase for the same length of time.

With regard to shell height the growth rate for the southern sample is greater (Fig. 3). Comparing the 1954 sample from southern California (3.0 ft.) with the Alaskan one, there is essentially no difference between the initial rates. However, subsequent segments of the curve show that the northern sample is decreasing its rate to a greater extent. The 1952 sample from southern California at the same intertidal level has a rate which is about 46% of the Alaskan group. The increase per thirty days for shell height was 0.85 mm. in 1952 and 0.69 mm. in 1954 for the sample from southern California, and 0.34 mm. for the northern one.

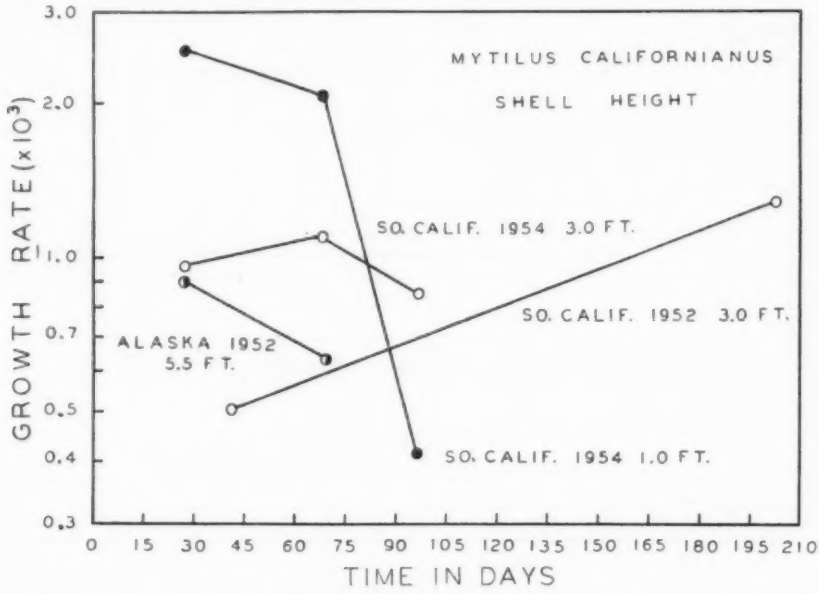

FIGURE 3. A comparison of the rate of growth in height of shell in populations of *Mytilus californianus* from Mount Edgecumbe, Alaska at one intertidal level, and Big Rock, California at two intertidal levels. Each point represents the mean k value for a sample of fifty or more individuals. The initial value is plotted on the day at which the second measurement was made.

It is evident from Figures 2 and 3 that the line drawn between the two k values (which represents the second and third measurements, May 25 to November 1, 1952) of both width and height of the populations from southern California (3.0 ft.) does not describe the conditions of linear increase in shell dimensions because of a lack of intermediate points. As a result, a more refined comparison might be made between the first two points on the curve (April 13 to May 25, southern California; June 26 to July 24, Alaska). Absolute increase in shell width for southern mussels was 0.86 mm. per thirty days, and for northern ones 0.32 mm. Increase in shell height for southern animals was 0.64 mm. per thirty days, and 0.43 mm. for northern ones. These latter values differ somewhat from the initial ones, be-

cause of the inclusion of more animals recovered at the second measurement. Relative rate (k) values have been determined for each individual of both populations, and then a mean rate calculated. Standard deviation for each rate was obtained, and a test was made to determine the significance of the difference between the two means (Alaska and southern California) when the population standard deviations are known. The mean rates for shell width are as follows: Alaska, $k = 0.00081$ (± 0.00055); southern California, $k = 0.00170$ (± 0.00166). The difference between these two means is significant ($P < 0.002$). Values for shell height are as follows: Alaska, $k = 0.00111$ (± 0.00083); southern California, $k = 0.00142$ (± 0.00107). The difference between these two means is insignificant ($P = 0.100$); such differences could occur by chance alone 10% of the time. A comparison of the mean rates (k) for length of shell between Alaskan and southern California mussels (3.0 ft.) shows them to be statistically significant ($P < 0.002$).

Comparison of the absolute increase per thirty days and/or the relative rate of increase of linear size between the 1952 and 1954 samples from southern California at the 3.0 ft. level is of value. This comparison points out the similarity of linear increase for two years in this population and is suggestive that the values obtained are characteristic of rates of increase of animals from this locality. These values also suggest that sea water temperatures were similar, which they were, and that the amount of plankton available was similar.

From these data, it would appear that the rate of size increase in shell length and width is greater in the sample of mussels from southern California than in that from Alaska, but the increase in shell height, though greater, cannot be said to be significantly different on these data. This means the shells are also growing differently shaped in the two populations.

Growth rate in vertically separated populations

During the spring and summer of 1954 not only were mussels studied at the 3.0 ft. tide level, but also at the 1.0 ft. level. The purpose of this was to determine the effect of intertidal height on the rate of linear increase of shell dimensions in this species. Reference to Figure 4 shows the differences in absolute increase in all shell dimensions per thirty days as it varies with tidal height. Data from Coe and Fox (1942) are included on this graph in order to illustrate the increase in mussels from La Jolla, California that were totally submerged. A comparison between populations located at the 1.0 ft. and the 3.0 ft. tidal levels from southern California indicates the great effect submergence has upon growth in this filter-feeding invertebrate.

Inspection of the data shows that the absolute increase in shell length at the 1.0 ft. level is over two times that at the 3.0 ft. level (2.3 mm./0.96 mm.), for a thirty-day period. With respect to width, the difference is not so great (1.3 mm./0.90 mm., 1952; 0.81 mm., 1954). An average of the last two values shows the mean increase of the higher level to be about 66% of the lower one. Absolute increase in height is about the same as the width relationship (1.2 mm./0.85 mm., 1952; 0.69 mm., 1954). An average of these values shows the 3.0 ft. animals to increase about 64% of the 1.0 ft. mussels.

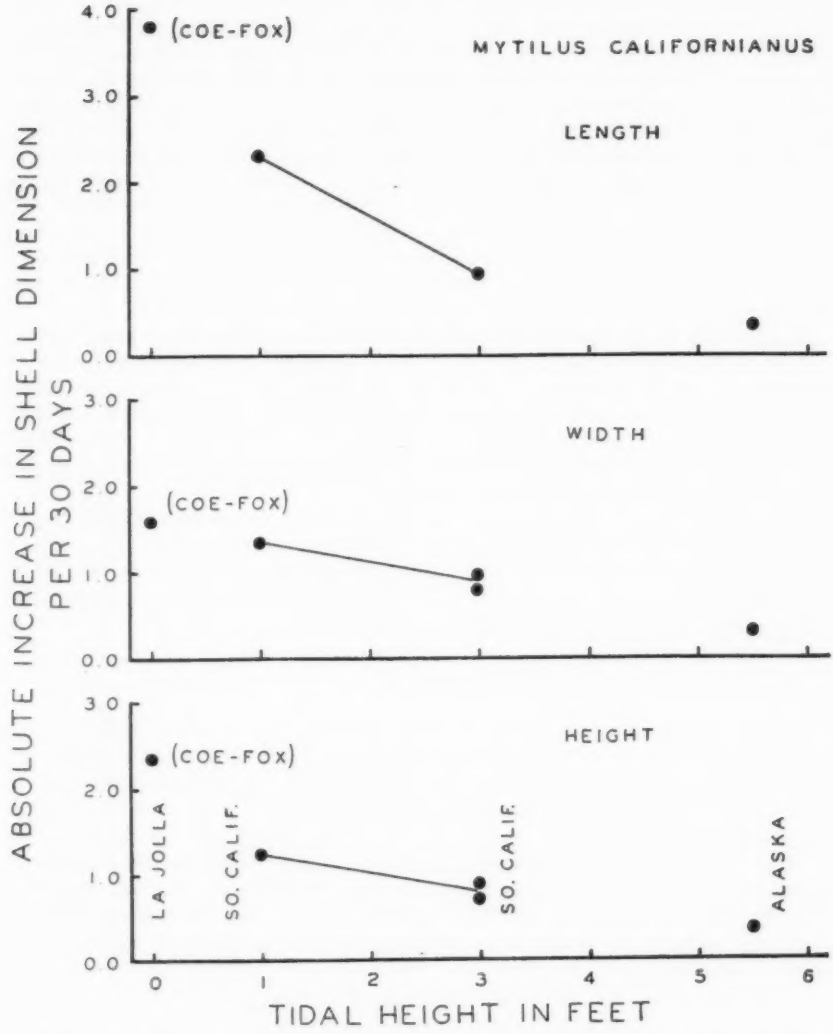

FIGURE 4. A comparison of the absolute increase in shell dimensions per thirty days as a function of intertidal height in populations of *Mytilus californianus* from La Jolla, California, Big Rock, California and Mount Edgecumbe, Alaska. Data for La Jolla mussels were obtained from Coe and Fox (1942, page 7, Table 1). Each point represents the mean increase for a sample of fifty or more individuals.

Discussion

It would be well to mention at the outset that the data presented by Coe and Fox (1942) for growth of mussels at La Jolla, California, under totally submerged conditions are in relative agreement with rates obtained at Big Rock, California, at the 1.0 and 3.0 ft. tide levels. In their paper, Coe and Fox (1942) present length measurements, and ratios from which width and height measurements may be obtained. Their ratios of $W/L = 0.37$ and $H/L = 0.5$ are not in agreement with the ones from Big Rock at either tidal level. However, there is no reason to believe that populations of mussels under entirely different ecological conditions should have the same growth ratios as ones submerged completely. Such mussels would conceivably compare only with subtidal populations of mussels, and even then growth might be much different.

The most obvious difference between populations of mussels from Big Rock at the 3.0 ft. and the 1.0 ft. tide levels is the degree of submergence per given period of time. Mussels located at the 3.0 ft. level were submerged about 58% of the time (14 hours per day), and ones from the 1.0 ft. level were submerged about 88% or twenty-one hours per day. It may be assumed that mussels obtain food in proportion to their access to it, *i.e.*, to their time of submergence. Rao (1954) has adduced good evidence for this. Then it may be expected that the food intake of the 1.0 ft. mussels compares with that of the 3.0 ft. ones as 88:58 or 100:66. If it is assumed that no difference existed in available food material per liter of sea water between the two populations at Big Rock, the difference between rates of linear increase in these two populations is partly explained.

The significance of vertical distribution as it affects rates of linear increase in species such as *Mytilus californianus* becomes apparent when mussels from southern California and Alaska are compared. Previously, it was stated that one population of Big Rock mussels was located at the 3.0 ft. tide level. These animals are able to filter approximately fourteen hours per day. Mussels from the population studied in Alaska were located at the 5.5 ft. tide level. They were able to filter about ten hours per day. The Big Rock mussels are submerged 1.4 times longer per twenty-four hours than the Alaskan ones. Even though the degree of submergence differs in the two populations, it is felt that mussels located at the 5.5 ft. level in Alaska are ecologically equivalent to ones from Big Rock at the 3.0 ft. tide level.

From values given by Rao (1953) pumping rates as a function of weight of soft parts for mussels from different latitudes may be determined. Weights of animals used were nearly the same (northern mussels have heavier shells relative to body weight), and by taking into account latitudinal acclimation, Big Rock mussels (16° C.) filter about two liters per hour, those from Alaska (10° C.), about one liter per hour. If the available organic matter per liter of sea water for northern and southern waters is the same, Big Rock mussels would filter $1.4 \times 2 = 2.8$ times as much food as the higher intertidal and only partially acclimated Alaskan sample. The relative growth rates (k) were found to be in the ratio 2:1 for length, 2:1, width and 1.3:1 for height. We may attempt to estimate the expected rate of linear growth in shell dimensions of Alaskan mussels if they were submerged to the same extent as the ones from Big Rock located at the 3.0 ft. level. Volume change must be calculated and this converted into linear increase. The ratio of food obtained is 2.8 and its cube root is 1.41. The value thus found

gives the ratio of expected fourteen hour to observed ten hour increase in length, width or height for Alaskan mussels. Also, this value (1.41) may be compared with the growth rate ratios given above. Considering the roughness of the estimate, the agreement is good and suggests that there is acclimation of growth rate.

If the interpretation presented is valid, and part of it is based upon the assumption and belief that the northern mussels are ecologically equivalent to lower intertidal southern ones, it could then be stated that even though relative rates in a population of *Mytilus californianus* from southern California are greater, mussels from Alaska, if chosen from a level with the same periods of submergence, would grow at approximately the same rate in spite of the lower temperature.

Summary

1. Rates of growth have been studied in populations of *Mytilus californianus* collected from southern California and southeastern Alaska. As nearly as possible natural rates of growth have been determined from populations in the field.

2. Length, width and height of the shell are used as criteria for determining rates of growth in this species. Comparisons of rates of growth between northern and southern populations are made by the use of relative rate (k) values.

3. Mussels were measured at the 1.0 ft. and 3.0 ft. tidal levels in southern California and the 5.5 ft. level in Alaska.

4. Rates of growth of *Mytilus* are found to be greater in the sample from southern California, comparing the 3.0 ft. level, than in that from Alaska. However, it was found that the southern California population is submerged for a greater period of time per week. The greater period of submergence and the greater weight of soft parts for a given shell size, recently shown by Rao (1953) for the southern population, mean a greater food intake in spite of acclimation of pumping rate. Attempts to correct for these factors suggest that the actual discrepancy in intrinsic growth rate is small or absent. In particular, Rao's findings give reason to believe that the heavier bodied southern mussels lay down no more shell length per unit of body weight than comparable northern ones.

5. Comparison of relative rates of growth in vertically separated populations of this species shows a large positive effect of submergence upon rate of increase of shell dimensions.

Literature Cited

Berry, S. S., 1953. On the supposed stenobathic habitat of the California sea-mussel. *Calif. Fish and Game*, **40**: 69–73.

Brody, S., 1945. Bioenergetics and growth. Reinhold Publishing Corp., New York.

Bullock, T. H., 1955. Compensation for temperature in the metabolism and activity of poikilotherms. *Biol. Rev.*, **30**: 311–342.

Coe, W. R., and D. L. Fox, 1942. Biology of the California sea-mussel (*Mytilus californianus*). 1. Influence of temperature, food supply, sex and age on the rate of growth. *J. Exp. Zool.*, **90**: 1–30.

Coulthard, H. S., 1929. Growth of the sea mussel. *Cont. Canadian Biol. and Fish.*, **4**: 123–136.

Dehnel, P. A., 1955. Rates of growth of gastropods as a function of latitude. *Physiol. Zool.*, **28**: 115–144.

Hatton, H., 1938. Essai de bionomie explicative sur quelques espèces intercotidales d'algues et d'animaux. *Annales de L'institut Océanographique*, **17**: 241–348.

HUXLEY, J. S., 1932. Problems of relative growth. Lincoln Mac Veagh, the Dial Press, New York.

MOORE, H. B., 1934. The biology of *Balanus balanoides*. I. Growth rate and its relation to size, season and tidal level. *J. Mar. Biol. Assoc.*, **19**: 851–68.

PROSSER, C. L., 1955. Physiological variation in animals. *Biol. Rev.*, **30**: 229–262.

RAO, K. P., 1953. Rate of water propulsion in *Mytilus californianus* as a function of latitude. *Biol. Bull.*, **104**: 171–181.

RAO, K. P., 1954. Tidal rhythmicity of rate of water propulsion in *Mytilus*, and its modifiability by transplantation. *Biol. Bull.*, **106**: 353–359.

RICKETTS, E. F., AND J. CALVIN, 1952. Between pacific tides. Stanford University Press, Stanford, California.

SEGAL, E., 1955. Microgeographic variation as thermal acclimation in an intertidal gastropod. Ph.D. dissertation, Univ. of California, Los Angeles.

SEGAL, E., K. P. RAO AND T. W. JAMES, 1953. Rate of activity as a function of intertidal height within populations of some littoral molluscs. *Nature*, **172**: 1108–1111.

WEYMOUTH, F. W., H. C. MCMILLAN AND W. H. RICH, 1931. Latitude and relative growth in the razor clam, *Siliqua patula*. *J. Exp. Biol.*, **8**: 228–249.

WIMPENNY, R. S., 1941. Organic polarity: some ecological and physiological aspects. *Quart. Rev. Biol.*, **16**: 389–425.

THE MIGRATORY HABITS OF THE MARINE GRIBBLE LIMNORIA TRIPUNCTATA MENZIES IN SAN DIEGO HARBOR, CALIFORNIA[1, 2]

MARTIN W. JOHNSON AND ROBERT J. MENZIES

Scripps Institution of Oceanography of the University of California, La Jolla, California

The isopod gribble *Limnoria* is a serious wood-boring pest in nearly all tropical, temperate, and boreal harbors where contamination has been established and where salinities are not markedly reduced by influx of fresh water. Typical examples of the ravishes of one or more species of this borer are offered by the findings of the San Francisco Bay Marine Piling Committee (Kofoid and Miller, 1927). During that survey only *Limnoria lignorum* (Rathke) was recognized, but Holthuis (1949) has shown that the species *L. quadripunctata* described by him as new was present in the area and had been confused with *L. lignorum*. Actually two species exist in San Francisco Bay, neither of which is *lignorum* (Menzies and Mohr, 1952).

Examination of collections from the San Diego region and elsewhere in California led Menzies (1951) to establish yet another species, *L. tripunctata* which has hitherto also been confused with *L. lignorum*.

Along the west coast of North America the geographic ranges of the three species of wood-boring *Limnoria* have been established as follows (Menzies, in manuscript): *Limnoria lignorum* (Rathke); Alaska to Point Arena, California. *Limnoria quadripunctata* Holthuis; California (41° N) to San Diego Bay. *Limnoria tripunctata* Menzies; warm parts of San Francisco Bay to Mazatlan, Mexico.

The seasonal activities of *L. lignorum* were studied at Friday Harbor, Washington, by Johnson (1935) who found that that species undergoes a pronounced seasonal swimming migration at which time new wood is attacked mainly during early spring. Sömme (1940) confirmed this type of activity for the same species in Norway and reported that a survey by Kramp in 1927 showed similar activity in Danish waters. Kramp's report, published in a Danish engineering journal, has escaped the attention of biologists in other parts of the world. He gives no figures of relative abundance or intensity of attack by *Limnoria lignorum* on new test blocks but states that at Hirtshals, Denmark, there was a pronounced maximum of attack in April. He concludes that the attacks result from periodic swarming of mainly adult individuals. Following the April swarming, the attacks occur in diminishing intensity during the summer.

It is of practical interest to know to what extent the findings relative to the northern species *L. lignorum* can be generally applied to the other species. With this in mind, the present study was designed to test *L. tripunctata*, a species of world-wide distribution in southern waters and the only one found in San Diego Bay.

[1] Contributions from the Scripps Institution of Oceanography, No. 829.

[2] Sponsored by the U. S. Navy Office of Naval Research, Project NR163-084, Contract Nonr-233(13).

Procedure

As a means of determining the migratory habits of *Limnoria tripunctata*, wooden test blocks were suspended freely in the water in the immediate vicinity of piling known to be heavily infested with *Limnoria*.

The test blocks consisted of 5 × 8 × 1 inch Douglas fir which provided about 106 sq. in. (684 cm.^{2}) of surface that could be examined by means of a low-power binocular microscope. After having been soaked in sea water free of borers for a period of 14 days, the blocks were attached to a rope line or stainless steel wire which was suspended from the pier and held taut by a concrete weight attached to the lower end, such that the weight hung free of the bottom. Test blocks thus rigged were located about one foot from infested piling so that they could be reached only by swimming migrating animals.

Two test stations were established in San Diego Bay: Station "A" at the U. S. Naval target repair base at Point Loma and Station "B" at the Johnson and Western Pier well within the bay near the Coronado ferry landing. Owing to reconstruction and repair there was some interruption at each station. The vertical position of the blocks and approximate days of exposure for each are given in Table I.

Table I

Position and exposure of test blocks

Station A

Aug. 12, '52–Aug. 13, '53		Aug. 13, '53–Feb. 15, '54		Apr. 14, '54–Mar. 14, '55	
Position of blocks	Days of exposure	Position of blocks	Days of exposure	Position of blocks	Days of exposure
Floating*	60				
Floating	30	Floating	30	Floating	30
6 ft. from bottom	30	6 ft. from bottom	60	6 ft. from bottom	60
4 ft. from bottom	30	4 ft. from bottom	90	4 ft. from bottom	90

* Zero tide datum is approximately 14.6 feet above bottom. The mean tidal range was approximately 4 feet.

Station B

Aug. 15, 1952–Aug. 13, 1953		Feb. 15, 1954–Mar. 14, 1955	
Position of blocks	Days of exposure	Position of blocks	Days of exposure
Floating**	30	5 ft. from bottom	30
2 ft. from bottom	30	4 ft. from bottom	60
		3 ft. from bottom	90
		2 ft. from bottom	120

** Zero tide datum is approximately 9 feet above bottom. Tidal range same as for Station A.

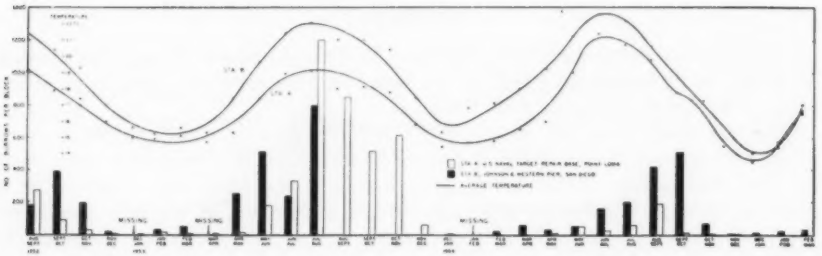

FIGURE 1. *Limnoria tripunctata.* Seasonal attack on test blocks exposed for successive 30-day intervals at Stations A and B, San Diego Bay from August, 1952 to March 14, 1955. Seasonal temperature included.

The intensity of settlement or attack on the blocks for a given period is taken to represent the intensity of migration for the period. It is determined by counting the number of burrows in the surface of the wood. Usually there are two animals, male and female, in each of the deeper burrows.

Surface temperature of the water was taken once each month at each station except for the period 15 August 1953 to 7 May 1954 when a continuous temperature recorder was in operation near Station A. Salinities in San Diego Bay fluctuate but little. The mean annual value is about 33.6‰.

Requirements for a study of this nature are: (1) nearby ample source of gribble infestation; (2) little or no interference resulting from repair or removal of piling; (3) safety from ship action, heavy seas or pilferage; (4) absence of adverse conditions due to low dissolved oxygen or low salinity.

These requirements were well met but for the already mentioned changes due to pier repair.

RESULTS

Seasonal Migrations

An examination of the severity of attack on the test blocks reveals that migration occurs to a greater or lesser degree throughout the year, but as in *L. lignorum,* there was each year a period of pronounced increase in migratory activity. In migrating the animals swim freely and a special preliminary study designed to reveal diurnal behavior in nature indicates that the greatest swimming activity takes place at night.

In Figure 1 is shown graphically the monthly seasonal fluctuations in migration between Aug. 12, 1952 and March 14, 1955 inclusive. The data used in compiling the figure were derived from the blocks submerged for successive 30-day periods with omissions as noted. Other periods of submergence, especially the 60-day periods, provided corroborative data on season of attack (Fig. 5) but the longer periods of submergence were designed mainly to provide information on rate of growth, sexual condition and reproduction.

The test blocks that were in place for 30-day periods beginning August 12, 1952, apparently caught mainly the declining portion of that year's migration.

In 1953 at Station A there was a period of active migration which extended from about the middle of May to the middle of November with a maximum during July–

August. This was also the peak 30-day period for Station B. This station was abandoned in August, 1953 when it was apparent that both stations at the various depth levels were showing a similar migratory picture; however, it was re-established when the reconstruction of piles at Station A forced a temporary abandonment of that station in January, 1954. By April, 1954 both stations were again studied until the completion of this phase of the work in March, 1955.

From the time of re-establishing of Station A in April, 1954, the attack on the blocks was relatively light but with a definite maximum occurring this time in August–September. The lighter attack may be attributed to a diminished source of infection as a result of replacement of infested piling, etc., with new pressure-treated creosoted timbers which left only a nearby infested stub and doubtless other undetected submerged wood, and some more distant infested structures. This also explains the low migratory figure shown in Figure 5 for the second year.

The main season of attack at Station B appears to be during August and September after which it diminishes rapidly to the low winter level.

The seasons of active attack on the blocks roughly coincide at the two stations, but it appears to have continued somewhat later at Station B in 1952 and began slightly earlier in 1953. The same trend is suggested in 1954. This difference may be correlated with the temperature. From Figure 1 it will be noted that a higher temperature prevailed throughout the year at Station B.

It has commonly been reported that the attack by *Limnoria lignorum* is not so great on floating or shallowly placed test blocks as it is on deeper blocks or near the bottom (Johnson and Miller, 1935; Sömme, 1940; Black and Elsey, 1948). This may be a factor of considerable importance in the relative rate of deterioration of floating or driven structures. However, our data from *Limnoria tripunctata* indicate that it shows no marked preference with respect to depth of settlement.

In order to better understand the migratory activity of *Limnoria* in light of its population biology an analysis was made of the seasonal composition of the population from the piling and from the test blocks. For this purpose the animals were studied as to size and, in the older specimens, also as to sex and reproductive condition.

Accurate measurements of the body length of *Limnoria* are usually difficult to obtain because of the natural tendency of the animals to assume a strongly bent position or for the segments to become more or less telescoped depending upon the type of preservative used or upon other factors such as ovarial development or injury. A practical substitute is therefore here made by using the pleotelson width at its widest point as an index of size. This has the advantage that we here deal with a flat rigid structure (the last segment of the body) that is not readily distorted. It has the further advantage that the size of animals in shallow burrows may often be determined without removing them from the burrow, and also the size of exuviae can be determined by this one fragment of the generally disarticulated cast.

Ten size categories can be established more or less clearly based on the width of the pleotelson.

In the literature dealing with *Limnoria*, the size index given is always in length; hence it will be useful here to provide Table II giving conversions of pleotelson widths to body lengths for the ten groups.

TABLE II

Limnoria tripunctata. Pleotelson width to body length. 180 animals measured

Size class	Mean pleotelson width, mm.	Body length, mm.		Remarks
		♂	♀	
1	0.24–0.26	0.8–1.2		Young lacking seventh pair of pereiopods
2	0.28–0.33	1.0–1.6		
3	0.35–0.40	1.1–1.6		Young with underdeveloped seventh pair of pereiopods
4	0.42–0.46	1.5–2.2	1.5–1.5	Sexual maturity reached by males
5	0.48–0.53	1.7–2.2	1.9–3.0	Females develop oostegites
6	0.55–0.60	2.1–3.0	1.8–3.0	Females become gravid
7	0.61–0.67	2.0–3.3	2.6–3.5	Includes majority of reproductive individuals, especially the females
8	0.69–0.74	2.5–3.4	3.0–3.8	
9	0.76–0.81	2.5–3.4	2.7–3.5	
10	0.82–0.86	2.5–3.2	2.5–3.8	Only few reach this size

An analysis of the population composition of a one-month test block and of old piling on the same date are given respectively in Tables III and IV. The results are quite typical, but for piling populations, there may be considerable variation in samples of one square inch in size taken adjacently in the same pile; hence, in most instances, counts were made in duplicate or triplicate.

A comparison of the percentage composition of the populations shown in Tables III and IV reveals that over 97% of the migrants are sexually mature animals of fairly large size, whereas on the piling, only about 28% are sexually mature, over 50% are newly hatched or immature animals, and 19% intermediate.

Analyses of piling samples taken monthly are given in Table V where the percentage composition of size classes are given and the mean calculated for three-month periods. Thus the variations are smoothed out to give seasonal means for

TABLE III

Limnoria tripunctata. Migrant population, Station A, Sept. 13, 1952. Numbers in size class, maturity, sex and per cent of total population (164) in test block of each month exposure

Size class	Immature	♀ (?) Young	♀ with oostegites	♀ gravid	♂
3	1				
4		2			1
5		1			16
6					12
7			21	7	25
8			12	10	11
9			18	11	10
10			2	2	2
% of Total	0.60	1.81	32.29	18.26	46.90

TABLE IV

Limnoria tripunctata. Piling population, Station A, Sept. 13, 1952. Numbers in size class, maturity, sex and per cent of total population in one square inch sample of infested wood with a total of 432 animals

Size class	7th pereiopod lacking	Immature	♀ (?) Young	♀ with oostegites	♀ gravid	♂
1	83					
2	36	39				
3		69	39			
4			39			8
5			5	4		10
6				10		7
7				7	13	23
8				8	10	7
9				4	7	4
% of Total	27.54	24.99	19.19	7.62	6.93	13.64

spring, summer, autumn, and winter. From this it appears that the piling population remains rather constant in its composition with some preponderance of the youngest class during the migratory months (June–November) and of the middle group during the winter (December–February) and relatively more adults in the spring (March–May) prior to heavy migration.

In the test block population presented in Table III, there is a slight predominance of females over males. It appears, however, from analyses of many block

TABLE V

Limnoria tripunctata. The mean percentage of size composition for monthly and seasonal periods for piling populations, 1952–1953

Size class	Spring			Seasonal mean	Summer			Seasonal mean
	March	April	May		June	July	August	
1–3	16	65.5	34	38.5	31.5	48	44	41.3
4–7	53.8	19.5	40.3	38	48	43.5	35	42.3
8–10	33.7	20	29	27.5	20.5	8.5	21	16.7
No. examined	403	783	792		545	449	300	
Size class	Autumn			Seasonal mean	Winter			Seasonal mean
	Sept.	Oct.	Nov.		Dec.	Jan.	Feb.	
1–3	69	34	47.5	50.3	17	53	33	34.3
4–7	16	51	40.3	35.8	63.5	34	54	50.7
8–10	15	15	12.3	14.1	19.6	13	13	15.2
No. examined	449	328	1282		557	153	44	

and piling samples, that the ratio of males to females on the piling is on an average about 1 to 1 for sexually mature specimens, whereas on test blocks it is often 1.3 to 1. This suggests that the males have a greater tendency to migrate than do the females, especially the younger females. Mating occurs after migration, the females becoming gravid some time after they have become established on the test block. This is evidenced by observations that (1) gravid females were found only in the longest, hence oldest burrows of the thirty-day test blocks, (2) here they occupied the blind end of the burrow while the male occupied the open end, (3) no gravid females were observed to occupy a burrow singly. This is in accord with the conclusions regarding the breeding habits of *Limnoria lignorum* (Kramp, 1927; Johnson, 1935; Sömme, 1940). Further evidence substantiating the opinion that migration precedes fertilization is found in Menzies' (1954) report which shows that females lack a sperm storage organ and the sperm produced by the male are relatively few in number and not grouped into spermatophores.

Sperm were found only in the oviduct of females which were moulting prior to deposition of eggs into the brood pouch. None were found in virgin or gravid females. Copulation probably precedes each brood production.

Factors Influencing Migration

Temperature

As shown in Figures 1 and 5 for *Limnoria tripunctata* and Figure 2 for *L. lignorum*, there is a certain correlation between the water temperature and the intensity of migration as indicated by severity of attack on the test pieces. The figures do, however, show a sharp difference in the maximum migratory period of the two species as related to the temperature curve and season of the year. *L. lignorum* reaches a peak of migratory activity on a rising temperature whereas *L. tripunctata* follows more nearly the seasonal trend of temperature with some tendency to lag behind. However, it should be pointed out that while the above appears to be typical for *L. lignorum*, at widely separated areas, data from Tromsösund, Norway, show a period of greatest migratory activity on a falling temperature. Sömme (1940) concluded that the season of migration varied with hydrographic conditions; hence each locality requires special investigation to determine the season of maximum attack on new wood. In a survey designed mainly to test the depth of attack in British Columbia waters, Black and Elsey (1948) indicate some variation in season of attack by *L. lignorum*. However, their data show that in areas such as Williams Head, Bentinick Island, and Esquimalt, which are not seriously affected by dilution, the main attack was during spring. The data used in Figure 2 A and B are derived from analysis of test blocks kindly provided us by the William F. Clapp Laboratories.

These differences in the two species may arise from specific differences in temperature requirements. Temperature *per se* may, however, not be the immediate cause of migration in either species of *Limnoria*. It is perhaps mediated through its effect on the rate of other biological activities such as growth, attainment of sexual maturity, and reproduction. Seasonal increases in these rates must lead to population pressure which is more acute at some seasons than at others.

Population pressure

In the study of *L. lignorum* at Friday Harbor (Johnson, 1935), it was hypothesized that migration results from overcrowding and approach of sexual maturity. Overcrowding is, of course, closely dependent upon the nature and availability of food supply and living space. *Limnoria,* like other wood borers, is unique in that it eats the substratum on which it lives. Limits to size and growth of the population are therefore set by the area of available wood. When conditions become intolerable due to population pressure, either a migratory movement must take place

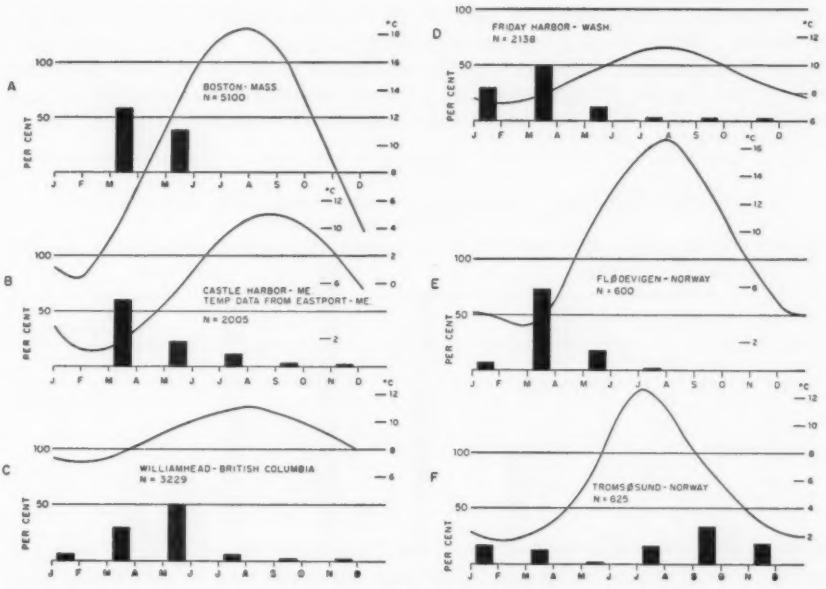

FIGURE 2. *Limnoria lignorum.* Percentage of seasonal attack in bimonthly periods in various localities. A and B data from William F. Clapp Laboratories; C from Black and Elsey, 1948; D from Johnson, 1935; E and F from Sömme, 1940. N = Yearly sum of animals on test blocks.

or the ratio of reproduction and growth to mortality must change. That the first takes place, we know, and there is some evidence also of the latter. The rate of growth, age of attainment of sexual maturity, and fecundity are then of prime importance in an understanding of migration.

Fecundity Related to Overcrowding

That there is a reduction of fecundity in a crowded population is indicated in Table VI. Here it is apparent that with increased size of the female, as shown by pleotelson width, there is also an increase in the number of eggs produced. This relationship is more evident for the test-block than for the piling population. It

will be noted also that the brood size of piling populations remains approximately one-half the size of that of the migrant population irrespective of size increase in the female. Thus it appears that an increased population density influences the brood size so that the rate at which young are added to a dense population may be only about one-half that at which they are added to a sparse population with plenty of wood for expansion. However, even with this adjustment, the population pressure must increase as the living space diminishes.

Fecundity Related to Temperature

The rate of development of eggs in the ovaries appears to be temperature-dependent. Brood-bearing females were found at Station A on test blocks which had been submerged for 30-day intervals all months of the year except the winter. In July and August, 1954, a brood was produced and hatched in 30 days at a temperature near 20.8° C. Test blocks submerged for 60 days showed gravid females

TABLE VI

Limnoria tripunctata. Comparison of brood size with size in females from old wharf piles and from 30-day test blocks

Size class	Piling			Test blocks		
	No./brood		No. gravid ♀♀ examined	No./brood		No. gravid ♀♀ examined
	Mean	Range		Mean	Range	
6	4.6	1–6	11	—	—	None found
7	5.8	1–12	25	8.9	4–18	35
8	4.0	1–12	28	10.8	1–18	79
9	5.1	1–12	34	12.4	1–22	95
10	7.0	1–12	3	10.5	4–21	21

throughout the year and hatched young most months of the year except during the spring of 1953 and in January and February, 1954. Station B yielded similar results for the 30-day exposures except that a brood was produced and hatched in less than 30 days in April and May (mean temperature 19.5° C.). The deposition of eggs and hatching of broods did not occur within 60 days at Station A during the winter or spring months of 1953–54 when temperatures ranged from 14.8 to 15.8° C. This suggests that with a temperature difference of only 5° C., it takes about twice as long to produce and incubate young during winter as it does during the summer.

It was observed that at least during the summer months, females with hatched young also had ripe ovaries and could incubate another brood soon after the emergence of the first batch from the pouch. Frequently young and their secondary or lateral burrows were observed in a burrow of a gravid female. In the test blocks exposed for three-month periods, the following maximum numbers of broods were observed: Station A, mid-November, 1953–mid-February, 1954 (mean temperature 14.8° C.) one brood; mid-July–mid-October, 1954 (mean temperature 20.6° C.) three broods; mid-August–mid-November, 1954 (mean temperature 17.4° C.) two

broods; Station B, mid-February–mid-May, 1953 (mean temperature 18.6° C.) two broods. Using size as a criterion, it appears that migrant females may have produced one or two broods prior to migration. This is indicated in Figure 3 which should be coordinated with Table II showing that females may become gravid in size class 6. Females in size class 10, the largest, were found only in migrant populations. Although migrants must frequently land on already heavily infested wood, the relative absence of the large females probably indicates that few, if any, re-establish themselves in dense populations. Thus the implication is that migration is largely an urge to seek less crowded conditions. Analysis of the population forming the basis of Figure 3 shows also that the summer and fall gravid migrants

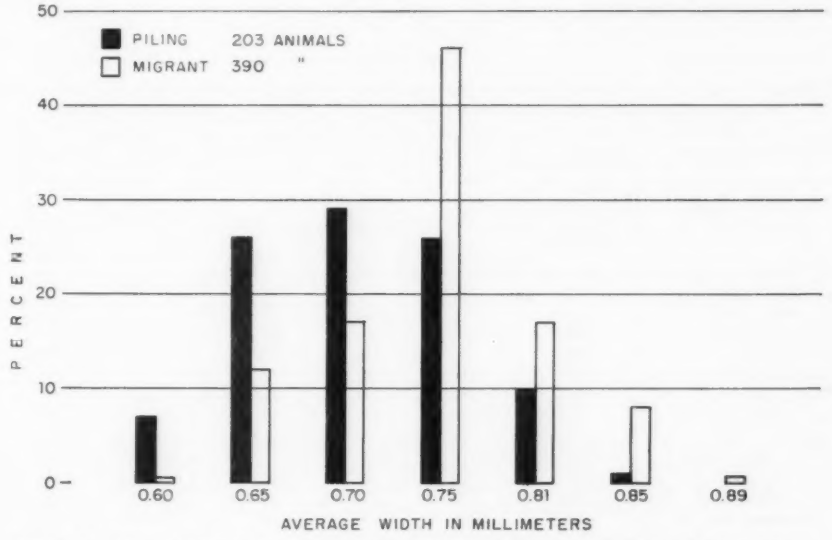

FIGURE 3. *Limnoria tripunctata.* Percentage size composition of gravid female populations.

average 0.04 mm. smaller than those of other seasons and the gravid females from piling average 0.05 mm. smaller, thus indicating that sexual maturity is reached at a smaller size (hence more rapidly) during the warmer months.

That reproduction in *Limnoria* is influenced seasonally by temperature is further indicated by a study of the "gravidity" which may be defined as the per cent of gravid females among the adult females occurring at any time in a piling population of maximum density (Table VII).

From Table VII, it seems evident that gravidity for this species reaches its peak somewhere between 17° and 19° C., declining at temperatures above and below these values. Gravidity appears to double between 14° and 18° C. In laboratory cultures only 28% became gravid at 24° C. The broods of specimens kept at 34° C. deteriorated, suggesting that such high temperatures may unfavorably affect brood production. At Beaufort, N. C., Coker (1923) reported no gravid females at

10° C. Mawatari (1950) reports that temperatures of 2–6° C. are lethal to *Limnoria lignorum* (probably *L. tripunctata*).

In contrast the northern species *Limnoria lignorum* appears to reach its peak gravidity at about 10° C., based on data from Johnson (1935). These data (assuming a 50-50 ratio of males to females) indicate that with environmental temperatures from 7.9° to 11.1° C. the gravidity figures varied from 22% to 79%, whereas at 7.5° C., the lowest temperature encountered, it was down to 14%. Sömme (1940) reports that *Limnoria* ceases to produce eggs at temperatures of 0° C. and below. In experiments she observed that the brood of a gravid female kept at 24.7° C. failed to develop after five days; at 14.8° C. and 6.3° C. development was apparently normal but at the lower temperature about twice the time was required as at an average of 13° C.

TABLE VII

Limnoria tripunctata. Seasonal gravidity (per cent gravid females of sexually mature females, 50/50 sex ratio assumed) in piling populations, Station A, 1952–1953. Figures based on gross size-class analyses of two to three one square inch samples of wood per month

Month sample collected	Mature animals of both sexes examined	Total females	Gravid females	% Gravid	Temperature °C.	
					Range	Approx. mean
January, 1953	96	48	6	12	15.5–14.8	15
February, 1953	124	62	34	55	14.8–14.6	15
March, 1953	289	44	83	57	14.6–15.0	15
April, 1953	109	54	31	57	15.0–15.5	15
May, 1953	220	110	68	62	15.5–16.3	16
June, 1953	133	66	53	80	16.3–18.3	17
July, 1953	44	22	12	55	18.3–19.2	18
August, 1953	53	26	28	100*	19.2–19.3	19
September, 1952	121	60	42	70	18.5–17.4	18
October, 1952	57	28	17	60	17.4–16.2	16
November, 1952	154	77	55	71	16.2–15.4	16
December, 1952	104	52	2	4	15.4–14.8	15

* In this case there were obviously more females than males in the sample.

Individual and population growth

The habits and life history peculiar to *Limnoria* make it possible to determine the growth rates of individuals and to follow the development of one or more broods in nature. To accomplish this, the test block (habitat) must have remained in the water a sufficiently long time to allow the migrants to incubate their broods and for the young to grow, but not so long that young of a second generation or new migrants confuse the picture. Development is direct and when the young emerge from the brood pouch, they immediately construct their own burrows within the walls of the parent burrow. The old female followed by the male continues burrowing deeper into the wood, so that subsequent broods released are separated more or less clearly from the previously released brood.

Thus up to the time that they may desert their burrows or have burrowed too

far from the parent burrow, the young produced on the block can readily be distinguished from young which might have migrated to the block. Even specimens which have become sexually mature, and no longer associated with the parent burrow can usually be distinguished from sexually mature young migrants to the blocks because they occur in significantly larger numbers than do the young (small) migrants.

Rate of Growth.

The growth rate of *Limnoria tripunctata* was determined by culturing individuals in the laboratory and from measurements of the growth of young on test blocks for two-, three-, and four-month intervals. The maximum growth rate seasonally as found in test block exposures is shown in Figure 4. An evident feature is the

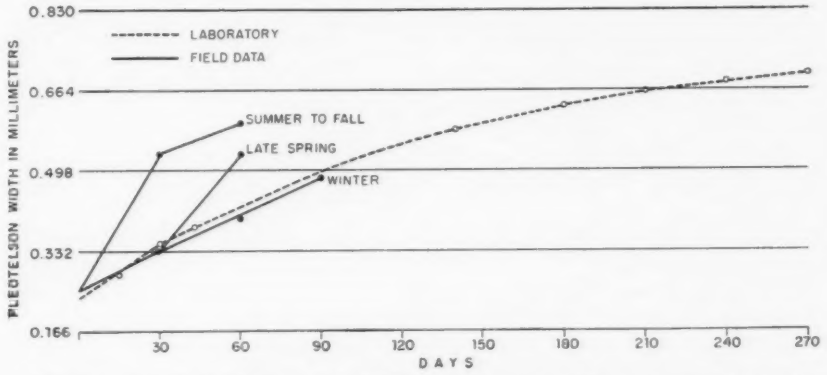

FIGURE 4. *Limnoria tripunctata.* Individual growth rate at different seasons: late spring, average temperature 19° C.; summer to fall, average temperature 22° C.; winter, average temperature 15° C.; laboratory culture average temperature 15° C. Measurements in field are from broods hatched on test blocks, those in laboratory, from individual specimens.

seasonal difference in growth rate of the young. Those emerging in winter require roughly twice as long to reach size class 5 (with 0.48–0.53 mm. pleotelson width) as do the spring young, and three times as long as the summer young. Thus increased crowding from growth of individual animals (distinguished from increased crowding resulting from addition of new individuals) would be greatest during spring and summer.

Population Growth.

Some idea can be had of the population growth by direct observation of the number and size of broods produced by migrant females during different portions of the year, namely on test blocks that were in place for successive 2-, 3- and 4-month periods throughout the year. In Table VIII summaries and calculations of probable annual rate of reproduction have been made, based on number of young observed in these 2- to 4-month periods.

Data from the test blocks show that a brood may be produced in a minimum of 25 days. The three- and four-month exposures indicate, however, that only one brood is produced during the winter, possibly three during the summer and at most only two during spring and fall. Thus a maximum of eight broods could conceivably result if a female can continue to produce successive broods at this rate. It has, however, been possible to follow the performance of individual females through a maximum of only three successive broods. Hence, it is not known whether or not a female that has produced three broods during the season of maximum production will continue to produce additional broods at the reduced rate at other seasons. Therefore, extrapolations must be considered with caution. Assuming an annual rate of eight broods with an average of 10 young per brood (Table VI) 100 females might produce a maximum of 8000 young. However, not all females in a population are gravid at any one time. The gravidity may be close to 100% during the mid-summer but drops as low as 4% during the winter (Table VII). An approximate mean gravidity for the year might be near 60% which would allow 100 females to produce only 4800 young per year at the rate shown for test board populations. Females in old piling populations appear to produce only about ½ this figure since their average brood size is only 5.

TABLE VIII

Limnoria tripunctata. Rate of production of young based on observed values for test block populations

Station	Duration of test block exposure	Dates of exposure	Total number of months	Number of young found per 100 mature females	Raw monthly rate	Adjusted* monthly rate	Adjusted* annual rate
A	2 months	Aug. 1952–Oct. 1953	12	380	31.6	63.2	758
A	2 months	Oct. 1953–Dec. 1954	12	717	59.7	119.4	1433
B	2 months	Feb. 1954–Feb. 1955	12	619	51.6	103.2	1238
A	3 months	April 1954–Jan. 1955	9	1009	112.1	149.1	1789
B	3 months	Feb. 1954–Jan. 1955	9	1251	139.0	185.3	2224
B	4 months	Feb. 1954–Feb. 1955	12	1585	132.0	165.0	1980
						Average annual rate	1570

* In order to approach the reproductive continuity that must occur on piling, one-month incubation time is allowed. Raw monthly rate on blocks of 2-month exposure are accordingly doubled, those of 3- and 4-month exposures are increased by ⅓ and ¼ of raw rate, respectively.

If the average annual realized reproductive performance of 100 sexually mature females is 1570 young as shown in Table VIII, the difference between this figure and the reproductive potential based on number and size of brood suggests the annual mortality on test blocks.

The reproductive rate given above may appear low compared with the potentialities suggested for *Limnoria lignorum* by Kramp (1927) who indicated four broods of 20–30 eggs per female per year. He, however, did not investigate the relative number of young resulting on the test blocks. Hence, no allowance was made for mortality. In Japan Shiino (1950) found only 1–12 eggs produced by *L. lignorum* (*tripunctata?*).

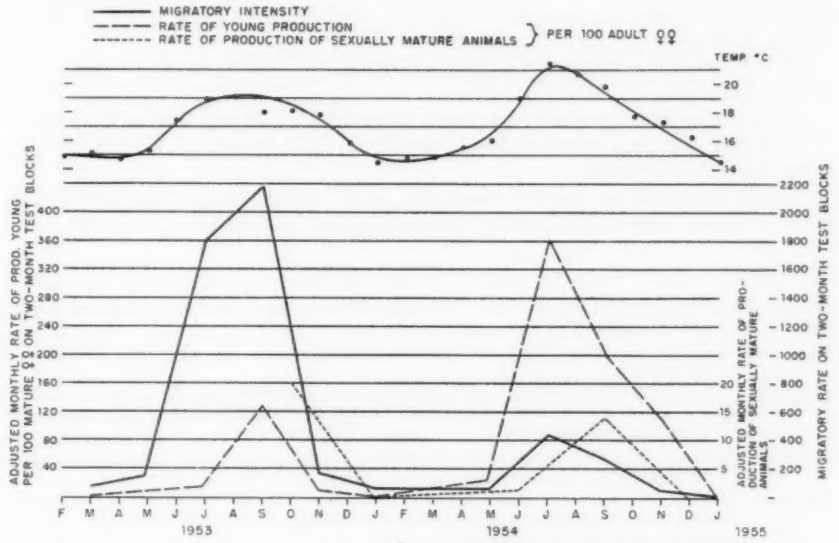
MIGRATORY INTENSITY
RATE OF YOUNG PRODUCTION
RATE OF PRODUCTION OF SEXUALLY MATURE ANIMALS
PER 100 ADULT ♀♀
TEMP °C
ADJUSTED MONTHLY RATE OF PROD. YOUNG PER 100 MATURE ♀♀ ON TWO-MONTH TEST BLOCKS
ADJUSTED MONTHLY RATE OF PRODUCTION OF SEXUALLY MATURE ANIMALS
MIGRATORY RATE ON TWO-MONTH TEST BLOCKS
F M A M J J A S O N D J F M A M J J A S O N D J
1953
1954
1955

FIGURE 5. *Limnoria tripunctata.* The relationship between monthly production rates and migration in two-month periods, Station A, San Diego Bay.

The relationship between monthly production rate and migration in San Diego Bay is shown graphically in Figure 5. Interpolated to the piling, these data indicate that a marked increase would have occurred there during the months of maximal migration.

SUMMARY

1. *L. tripunctata* undergoes a definite seasonal migratory period which reaches its height approximately during the season of highest water temperatures. This differs from the earlier migratory season usually shown for *L. lignorum*.

2. The attack on new wood is about equally severe on floating and submerged blocks.

3. A comparison of the percentage composition of the 30-day test-block populations shows that about 97% of the migrants are sexually mature animals as con-

trasted with piling populations where 50% consisted of newly hatched immature animals and only about 28% sexually mature. The piling population remains fairly constant in its composition.

4. None of the females is gravid at the time of migration. Pairing takes place after establishment on the test blocks.

5. As with *L. lignorum* the factors influencing migration are probably a combination of approach to a reproductive phase and population pressure due to overcrowding in old established populations.

6. Water temperature is important in influencing migration in that the higher temperatures hasten the rate of growth and attainment of sexual maturity, production of eggs, and shorten the incubation period. Gravidity (per cent gravid females) in *L. tripunctata* evidently reaches its peak between 17° and 19° C. as contrasted with about 10° C. for *L. lignorum*.

7. The brood size of piling populations is about ½ that found in uncrowded test-block populations.

8. A single female probably produced three or more broods during the year.

9. Using size as a criterion, most migrant females appear to have produced a brood prior to migration.

10. In extrapolating the test-block data to the piling populations, there is indicated a marked population increase during the months of maximal migration.

LITERATURE CITED

Black, Edgar C., and G. R. Elsey, 1948. Incidence of wood-borers in British Columbia waters. *Bull. Fish. Res. Bd. Can.*, **80**: 1–20.

Coker, R. E., 1923. Breeding habits of *Limnoria* at Beaufort, N. C. *J. Elisha Mitchell Sci. Soc.*, **39**: 95–100.

Holthuis, L. B., 1949. The Isopoda and Tanaidacea of the Netherlands, including the description of a new species of *Limnoria*. *Zool. Mededelingen Reiksmuseum Van Natuurlijke Hist. te Leiden*, **30**: 163–190.

Johnson, Martin W., 1935. Seasonal migrations of the wood-borer *Limnoria lignorum* (Rathke) at Friday Harbor, Washington. *Biol. Bull.*, **69**: 427–438.

Johnson, Martin W., and Robert C. Miller, 1935. The seasonal settlement of shipworms, barnacles and other wharf-pile organisms at Friday Harbor, Washington. *Univ. of Washington Pub. in Oceanography*, **2**: 1–18.

Kofoid, C. A., and R. C. Miller, 1927. Biological Section, Final Report of the San Francisco Bay Marine Piling Committee (published by the Committee), pp. 188–343.

Kramp, P. L., 1927. Undersögelser over Paeleorm og Paelekrebs ved Hirtshals. *Ingeniören*, **36**: 329–340.

Mawatari, Shizuo, 1950. Biological and industrial study of marine borer problem in Japan. Studies on the aquatic animals of Japan (1), pp. 9–12, 45–124. In Japanese with English summary.

Menzies, Robert J., 1951. A new species of *Limnoria* (Crustacea: Isopoda) from Southern California. *Bull. So. California Acad. Sci.*, **50**: 86–88.

Menzies, Robert J., 1954. The comparative biology of reproduction in the wood-boring isopod crustacean *Limnoria*. *Bull. Mus. Comp. Zool.*, **112**: 364–388.

Menzies, R. J., and J. L. Mohr, 1952. The occurrence of the wood-boring crustacean *Limnoria* and of Nebaliacea in Morro Bay, California. *Wasmann J. Biol.*, **10**: 81–86.

Shiino, Sueo M., 1950. The marine wood-boring crustacea of Japan. I. Limnoriidae. *Wasmann J. Biol.*, **8**: 333–358.

Sömme, Olaug M., 1940. A study of the life history of the gribble *Limnoria lignorum* (Rathke) in Norway. *Nytt. Mag. for Naturvidenskapene*, **81**: 145–205.

THE OCCURRENCE OF PORPHYRIN IN THE PLANARIAN [1]

EDITH KRUGELIS-MACRAE

Department of Zoology, University of California, Berkeley 4, California

During a study of electrophoretic properties of protein extracts of planarians, there was observed a substance characterized by a bright red fluorescence under ultra-violet light and a high electrophoretic mobility. The fluorescence suggested the substance could be a porphyrin or a related compound. Recent reviews in comparative animal biochemistry, although treating the occurrence of porphyrins, give no information as to the occurrence of porphyrin in planarians (Baldwin, 1939; Florkin, 1947; Prosser *et al.*, 1950).

An investigation of this substance with regard to some of its chemical properties identifying it as a porphyrin will be reported in this paper, and the possible biological significance will be discussed.

Materials

The biological material used was the planarian, *Dugesia dorotocephala;* the species is distinguished from related species by its large size (20–30 mm.) and dark pigment (Hyman, 1951). The planarians were collected in a pool on the Berkeley campus of the University of California. The water of the pool was covered by a dense growth of water fern among which the planarians were found. The temperature of the water varied between 14° and 16° C. during the summer when the animals were collected and studied. The animals were used for experiments on the next day or the same day as collected, unless stated otherwise.

Experimental Data

1. Preparation of extracts and preliminary results

The planarians were washed with distilled water and then homogenized in a Potter-type homogenizer. Distilled water was added to wash out the thick suspension. This water extract was centrifuged at low speed (about 500 rpm). The supernatant was discarded; the dark residue was resuspended in distilled water and centrifuged again. The washed residue was suspended in 0.01 *M* sodium carbonate at pH 11.0, in which solution almost all the residue dissolves. This was centrifuged and the pale brownish supernatant contained the substance fluorescing red under ultra-violet light. The original supernatant of the water extract was examined under ultra-violet; this possesses no red fluorescence but rather a pale gray fluorescence. No red fluorescence could be produced in the water-extracted supernatant by adding sodium carbonate up to the pH at which the dissolved residue

[1] This investigation was supported by a research grant (C-2410 M and G) from the National Cancer Institute, of the National Institutes of Health, Public Health Service.

fluoresces. This suggested that there was a red fluorescent substance bound to or adsorbed on the sedimentable granules that was not eluted with water but was extracted or eluted with aqueous sodium carbonate at pH 11.0.

The absorption spectrum of the residue dissolved in Na_2CO_3 was ascertained using a Beckman spectrophotometer. The spectrum showed a sharp absorption band near 400 mμ, as well as several weaker bands at the longer wave-lengths. The sharp absorption near 400 mμ is characteristic of the porphyrin ring and is found for all porphyrins regardless of their side chains. This band is termed the Soret band. The spectrum of the substance in Na_2CO_3 served only for its identification as a porphyrin. Using HCl as a solvent revealed the spectrum more clearly and permitted comparison with absorption maxima of porphyrins as reported in the literature; these maxima will be presented later in the paper.

2. Solubility study

The solubility of the porphyrin in various solvents was determined by suspending the water-insoluble residue in the solvent; the porphyrin was considered soluble if ultra-violet light [2] revealed red fluorescence in the solution. By this criterion, the porphyrin was found to be soluble in Na_2CO_3 (0.01 *M*), KOH (0.1 N), NH_4OH (10%), HCl (5% and 25%), and ethyl acetate (in acetic acid); it was insoluble in ethyl alcohol (95%), acetic acid (1%), chloroform, acetic-ether and ether. The fact that the porphyrin is insoluble in ether places it in the category of ether-insoluble porphyrins. This eliminates the possibility that the porphyrin in question is a protoporphyrin, mesoporphyrin, deuteroporphyrin or coproporphyrin, all of which are ether-soluble. The insolubility in ether and its solubility in ethyl acetate (in acetic acid) are characteristic of uroporphyrins, which are porphyrin octacarboxylic acids unextractable in ether or chloroform unless the carboxylic acid groups are esterified (Lemberg and Legge, 1949).

The methyl ester of the porphyrin was prepared by adding an acid solution of the porphyrin to cold methyl alcohol saturated with HCl, and allowing the solution to remain at 5° C. for several days. The porphyrin ester can then be extracted with chloroform. A hydrolysis of the ester can be achieved by adding 25% HCl to the methyl ester in chloroform and allowing the mixture to stand at 5° C. for several days. The red fluorescence of the porphyrin then appears in the top aqueous acid layer. This procedure was used for purification of the porphyrin for absorption spectrum studies.

3. Absorption spectrum study

The absorption spectrum of the planarian porphyrin in 5% HCl revealed maxima at I 600 mμ, II 555 mμ, III 495 mμ, and IV 406 mμ; order of intensity IV, III, II, I. When the porphyrin was dissolved in 25% HCl the maxima were I 598 mμ, II 553 mμ, III 496 mμ, and IV 409 mμ (see Fig. 1). The maxima reported by Grinstein, Schwartz and Watson (1945) for purified uroporphyrins in 25% HCl are: uroporphyrin (204° C. melting point) I 597 mμ, II 553 mμ, III 410 mμ; uroporphyrin (284° C. melting point) I 595 mμ, II 552 mμ, III 408 mμ.

[2] The source of ultra-violet radiation was a 6 watt GE360 Black Light lamp which produces long wave (3660 Å) ultra-violet. The lamp was supplied by Vogel Luminescence Corp.

The absorption spectrum of the methyl ester of the porphyrin in chloroform had maxima at I 626 mμ, II 582 mμ, III 575 mμ, IV 535 mμ, V 502 mμ, VI 408 mμ. The maxima reported by Grinstein *et al.* (1945) for the methyl ester of uroporphyrin in chloroform are I 626 mμ, II 581.5 mμ, III 570.5 mμ, IV 536 mμ, V 501.5 mμ, VI 408 mμ.

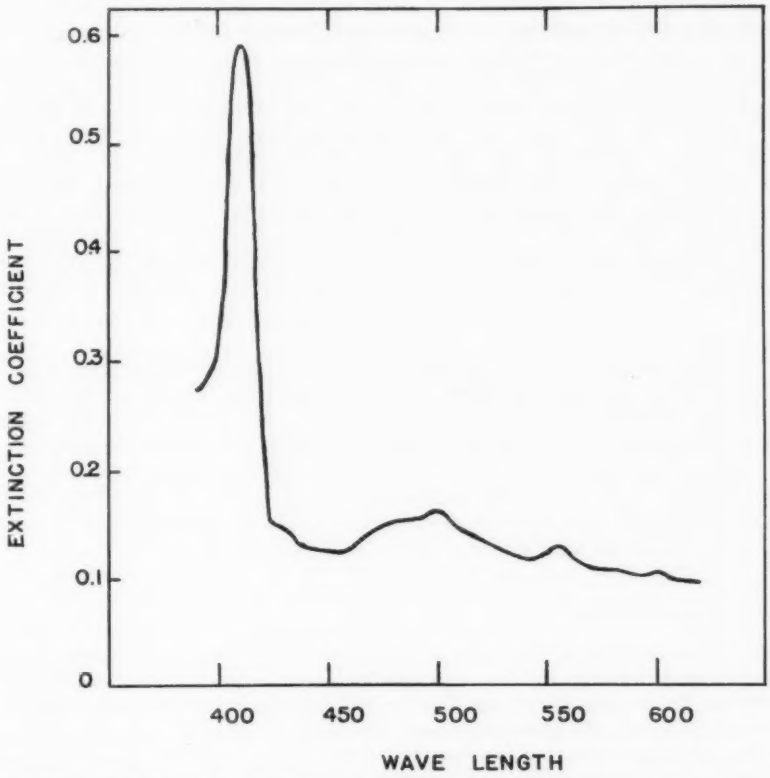

FIGURE 1. The absorption spectrum of the planarian porphyrin extracted in 25% HCl directly from water-insoluble granules is shown. The extinction coefficient ($\log \frac{I_o}{I}$) is plotted against the wave-length (in millimicrons).

After hydrolysis of the methyl ester with 25% HCl, the purified planarian porphyrin had maxima at I 596 mμ, II 553 mμ, (511 mμ) III 409 mμ; order of intensity III, II, I. After purification by esterification and hydrolysis, the absorption at 496 mμ is removed; this suggests that the absorption at 496 mμ was due to another substance, perhaps a related compound.

There is considerable similarity between the absorption spectrum of the por-

phyrin from planarians and the uroporphyrin isolated by Grinstein *et al.* (1945) from urine of patients with a congenital porphyria which is characterized by excretion of large quantities of uroporphyrin.

4. Paper electrophoresis

The mobility of the porphyrin was observed with paper electrophoresis using a somewhat modified technique of Kunkel and Tiselius (1951). Whatman 3MM filter paper was held between glass plates and dipped at the ends into electrode vessels containing Na_2CO_3 (0.01 *M*), $\mu = 0.03$, pH 11.0. The porphyrin solution was placed on the paper at the cathode end 17 cms. from the cathode and 40 cms. from the anode. The run was made at room temperature using 200 volts and 3–5 milliamperes. Serum albumin stained with brom phenol blue was applied parallel to the porphyrin for comparison of relative mobilities. Both the stained albumin and the red fluorescent spot of the porphyrin moved as discrete areas towards the anode. The location of the anterior edge of the two moving areas was noted at intervals. The distance moved for porphyrin and albumin, respectively, was 5.7 cms. and 3.8 cms. after one hour; 9.7 cms. and 6.0 cms. after two hours. The ratios of their relative mobilities were 1.45 at one hour and 1.6 at two hours.

To locate protein at the completion of the run, the paper was immersed in 10% $HgCl_2$ in 95% ethyl alcohol, stained in brom phenol blue (0.1% in 95% ethyl alcohol) for one hour, destained in 1% acetic acid. No protein stain was observed at the final location of the red fluorescence, although the protein staining revealed a protein of low mobility not associated with the red fluorescence under these conditions of electrophoresis. The protein may have dissociated itself from the porphyrin during the electrophoresis or may be a separate protein found in the residue and extracted by the same alkaline solvent as the porphyrin.

5. Paper chromatography

The identification of the planarian porphyrin was checked by paper partition chromatography using a lutidine-water system described by Nicholas and Rimington (1951). There is a linear relationship between R_F values and the number of carboxyl groups in the porphyrins and this can be used to identify the porphyrin.

The porphyrin was extracted in 2 N NH_4OH or 1% HCl and applied in the solvents to pieces (12 × 15 cms.) of Whatman No. 1 filter paper along a base line 2 cm. from the lower edge. The chromatographs were run in the dark at 21° C. in a lutidine-water system containing 40% water by volume. The position of the spots after development was determined by their fluorescence in ultra-violet light.

The porphyrins run were the planarian porphyrin assumed to be a uroporphyrin and a known sample of hematoporphyrin. R_F values were calculated and compared. Under these experimental conditions, the R_F value of the hematoporphyrin was between 0.7 and 0.8; the R_F value of the planarian porphyrin was between 0.01 and 0.1. The R_F values reported by Nicholas and Rimington (1951) for two-carboxyl porphyrins (such as hematoporphyrin) were about 0.8 and for eight-carboxyl porphyrins (uroporphyrin) were about 0.1 under experimental conditions similar to

those used in these experiments. This suggests that the planarian porphyrin contains eight carboxyl groups and may be uroporphyrin.[3]

6. Content of porphyrin in planarians

To determine whether porphyrin content varied from one planarian to another, different numbers of animals of approximately the same size were collected and the relative amount of porphyrin determined. The extinction coefficient ($\log \frac{I_o}{I}$) at the maximum absorption, 408 mμ, was used as the measure of porphyrin content. Since planarians vary in size, it is necessary to relate the porphyrin to some unit of planarian size or weight. Wet weight was chosen as the most convenient measure. Since the porphyrin is insoluble in ethyl alcohol, this can be used as a means of killing or fixing the animals and thus facilitating wet weight determinations. Before extraction of the porphyrin, a known number of planarians was placed in a tared watch glass, the animals were immediately killed with 95% ethyl alcohol, and filter paper was applied to remove excess moisture before weighing. After weighing, the planarians were homogenized in water, centrifuged, the residue extracted

TABLE I

Number of planarians	Extinction at 408 mμ	Total wet weight (mg.)	Ratio (E/weight)
2	0.081	13.7	0.0059
4	0.256	41.6	0.0061
6	0.335	51.7	0.0064
8	0.412	60.9	0.0067
10	0.482	71.2	0.0067

in a constant volume (3 ml.) of 25% HCl, and the extinction coefficient determined. The porphyrin coefficient was stated in arbitrary units as the extinction at 408 mμ per total wet weight. The data are presented in Table I. Under similar physiological conditions, the unit of porphyrin per planarian of a given wet weight may be a constant.

Preliminary experiments indicate that the porphyrin is also present in animals which had been starved in the laboratory for one, two and four weeks. Porphyrin is present in regenerating and regenerated animals. The porphyrin is not confined to any one region of the animal; it is distributed throughout the animal although the distribution may not be uniform along the length of the animal.

7. Visual demonstration of porphyrin

A visual demonstration of porphyrin within the planarian can be made by exposing animals to ultra-violet light after they have been immersed in 95% ethyl

[3] A sample of the planarian porphyrin was analyzed recently in the laboratory of Dr. Samuel Schwartz of the Department of Medicine at the University of Minnesota. More refined techniques of chromatography on calcium carbonate revealed two red fluorescent zones. Paper chromatography showed the presence of coproporphyrin and a porphyrin which behaves like uroporphyrin. The author wishes to thank Dr. Samuel Schwartz, as well as Dr. Marie Berg and Mr. Micheal Keprios for their analysis of this material.

alcohol. One can then observe the whole planarian emitting a red fluorescence. No definite fluorescence could be detected in the living animal, although the water-insoluble sedimentable granules do show some fluorescence without further chemical treatment. The alcohol removes from the animal a substance or substances which may obscure the red fluorescence. This substance is yellow in solution, has a yellow fluorescence, and is soluble in organic solvents such as ethyl alcohol, methyl alcohol, acetone, chloroform and pyridine. Any of these organic solvents can remove the yellow substance from the whole animal and allow the red fluorescence of the porphyrin to be observed. This yellow substance is probably a lipochrome or carotenoid, and requires further investigation.

Discussion

Uroporphyrins were first described by Fischer in 1915 and since then much work has been done to characterize and describe this substance (see review by Lemberg and Legge, 1949). The main source of uroporphyrin has been the urine of patients having congenital porphyria (Waldenström *et al.*, 1935; Grinstein *et al.*, 1945). In the case of porphyria the occurrence of uroporphyrin is considered to be a metabolic error. However, in the case of the planarian, this porphyrin seems to be found in all individuals and may occur as a physiological porphyria, as Turner (1937) described the case in the fox-squirrel where uroporphyrin occurs normally in the bones. Uroporphyrin is found normally in small amounts in the zones of calcification of foetal bones and it is found in larger amounts in the bones and teeth of congenital porphyria cases in man and other mammals (Rimington, 1955). Uroporphyrin occurs in the *Pteria* mussel shell (Fischer and Haarer, 1932; Nicholas and Comfort, 1949) and occurs as a copper complex in the flight feathers of the African turacos (Fischer and Hilger, 1924). In these cases the uroporphyrin seems to be stored in metabolically rather inert tissues, except perhaps for foetal bones. This suggests that the uroporphyrin may be an end-product of chemical events, perhaps of calcification in some cases. Uroporphyrin has been postulated to be in the biosynthetic pathway leading to the formation of haem, a porphyrin containing substance vital to higher organisms, but recent studies suggest that uroporphyrin may be a side reaction and not involved in the biosynthetic pathway to haem (see review of Rimington, 1955). Thus uroporphyrin may be an end product whenever it occurs, either in pathological urine, bones, teeth, feathers, shells or in planarians. However, in the case of the planarian, the porphyrin is distributed rather generally and is not stored in inert tissue. This raises the question whether there may not be some biological significance for the planarian porphyrin.

Loeb (1893) and Hesse (1897) had independently observed that the movements of planarians were influenced by light. Parker and Burnett (1900) extended these observations and measured the speed of movement of planarians away from light; they found that animals with and without eyes responded so as to move away from light but the eyeless ones at a somewhat slower rate than those with eyes. It has also been shown that planarians perceive ultra-violet radiations and produce a negative reaction; the behavior is the same for normal and eyeless specimens (Werner, 1926). Short exposures to ultra-violet and to direct sunlight are fatal to planarians (Merker and Gilbert, 1932).

The question is whether this sensitivity and response to ultra-violet may wholly or in part perhaps be due to the presence of porphyrins which are strongly photoactive and efficient in absorbing light around 4000 Å. This response may be related to the photosensitizing action of porphyrins discovered in 1909 by Hausmann (as reported by Lemberg and Legge, 1949). He found that experimental animals and humans injected with porphyrin solutions and then exposed to sunlight or ultra-violet light, showed a skin sensitization. Also it is known that human individuals suffering from congenital porphyria show a marked light sensitization of the skin (Lemberg and Legge, 1949). In planarians the light sensitization may have a normal function to produce the avoidance of light.

The author wishes to acknowledge the technical assistance of Miss Emily Martin.

Summary

1. A porphyrin was extracted from the water-insoluble residue of homogenates of the planarian, *Dugesia dorotocephala*. The porphyrin extract is characterized by a red fluorescence under ultra-violet light.

2. The solubility properties of the porphyrin, the absorption spectra of the porphyrin and its methyl ester, and paper chromatographic behavior show a similarity to those described for uroporphyrin.

3. The amount of porphyrin seems to be a constant per planarian of a given wet weight, when the animals are under the same physiological condition. The porphyrin is also found in starved and in regenerating animals.

4. The whole planarian emits a red fluorescence under ultra-violet light if the animal is first immersed in one of several organic solvents which remove a lipochrome or carotenoid substance.

5. The light sensitivity which has often been described for these animals may to some degree be due to the presence of the porphyrin.

LITERATURE CITED

Baldwin, E., 1939. Comparative biochemistry. Cambridge University Press, Cambridge.

Fischer, H., 1915. Über das Urinporphyrin. *Zeitschr. f. physiol. Chem.*, **95**: 34–60.

Fischer, H., and E. Haarer, 1932. Über Uroporphyrin aus Muschelschalen. *Zeitschr. f. physiol. Chem.*, **204**: 101–104.

Fischer, H., and J. Hilger, 1924. Zur Kenntnis der naturlichen Porphyrine. *Zeitschr. f. physiol. Chem.*, **138**: 49–67.

Florkin, M., 1947. L'évolution biochimique. Masson et Cie., Paris.

Grinstein, M., S. Schwartz and C. J. Watson, 1945. Studies of the uroporphyrins. *J. Biol. Chem.*, **157**: 323–344.

Hesse, R., 1897. Untersuchungen über die Organe der Lichtempfindung bei niederen Thieren. *Zeitschr. wiss. Zool.*, **62**: 527–582.

Hyman, L. H., 1951. The invertebrates: Platyhelminthes and Rhynchocoela. Vol. II. McGraw-Hill Book Co., New York.

Kunkel, H. G., and A. Tiselius, 1951. Electrophoresis of proteins on filter paper. *J. Gen. Physiol.*, **35**: 89–118.

Lemberg, R., and L. W. Legge, 1949. Hematin compounds and bile pigments. Interscience Publishers, New York.

Loeb, J., 1893. Ueber künstliche Unwandlung positiv heliotropischer Thiere in negativ heliotropische und umgekehrt. *Arch. f. Ges. Physiol.*, **54**: 81–107.

MERKER, E., AND H. GILBERT, 1932. Die Widerstandsfähigkeit von Süsswasserplanarien in Ultra violettreichem Licht. *Zool. Jahrb. Abt. Allg. Zool.*, **50**: 479–556.

NICHOLAS, R. E. H., AND A. COMFORT, 1949. Acid-soluble pigments of molluscan shells. *Biochem. J.*, **45**: 208–210.

NICHOLAS, R. E. H., AND C. RIMINGTON, 1951. Paper chromatography of porphyrins; some hitherto unrecognized porphyrins and further notes on the method. *Biochem. J.*, **48**: 306–309.

PARKER, G. H., AND F. L. BURNETT, 1900. The reactions of planarians with and without eyes to light. *Amer. J. Physiol.*, **4**: 373–385.

PROSSER, C. L., D. W. BISHOP, F. A. BROWN, JR., T. L. JAHN AND V. J. WULFF, 1950. Comparative animal physiology. W. B. Saunders Co., Philadelphia.

RIMINGTON, C., 1955. Porphyrins. *Endeavour*, **14**: 126–135.

TURNER, W. J., 1937. Studies on porphyria. I. Observations on the fox-squirrel, Sciurus niger. *J. Biol. Chem.*, **118**: 519–530.

WALDENSTRÖM, J., H. FINK AND W. HOERBURGER, 1935. Über ein neues bei der akuten Porphyrie regelmahig vorkommended Uroporphyrin. *Zeitschr. f. physiol. Chem.*, **233**: 1–9.

WERNER, O., 1926. Reizphysiologische Untersuchungen an Planarien in ultravioletten Lichte. *Zool. Jahrb. Abt. Allg. Zool.*, **43**: 41–68.

OXYGEN CONSUMPTION OF WHOLE INSECTS AND INSECT HOMOGENATES [1]

DANIEL LUDWIG AND MARY C. BARSA

Department of Biology, Fordham University, New York 58, New York

The alteration of enzyme activity by cell rupture has been extensively studied with vertebrate tissues, but very little work has been done with those of invertebrates. Bodine and Lu (1951) found that homogenization of both diapause and actively growing embryos of the grasshopper, *Melanoplus differentialis*, reduced oxygen consumption by approximately 65 per cent. Their results agree with the 60 to 70 per cent decrease obtained with vertebrate tissue (De Robertis, Nowinski and Saez, 1950). They concluded that 65 per cent of the oxygen uptake of intact embryos is due to structure and the remainder to the basic chemical composition of the cells.

In the present study, the oxygen consumption of homogenates of the Japanese beetle, *Popillia japonica*, and of the mealworm, *Tenebrio molitor*, was determined for each day of embryonic development at 30° C. A comparison was then made with the oxygen consumption of whole eggs. They lack a diapause and thus differ from the eggs of the grasshopper, *M. differentialis*. A similar comparison was made with whole insects and their homogenates in the larval stage, and for each day of the pupal stage of the mealworm at 30° C. Finally, a study was made of the effect of different homogenate concentrations on the oxygen consumption of larvae and pupae of the mealworm.

Material and Methods

Japanese beetle eggs were collected daily and placed on the surface of moist, firmly-packed soil in one-ounce metal salve boxes. Eggs of the mealworm were collected daily from beetles kept in white flour. They were stored in a desiccator over a saturated solution of NaCl (relative humidity, 75 per cent). Eggs of both species were incubated at 30° C.

Larvae of the mealworm, obtained from a mixed culture maintained at room temperature, were used in these experiments. Prepupae were collected from the same culture and placed at 30° C. They were examined daily and the newly molted pupae were placed in beakers and kept at 30° C. In this manner, pupae of known ages (within 24 hours) were obtained.

All homogenates were made in 0.03 molar phosphate buffer, adjusted to a pH of 7.4. The tissue was homogenized for one minute in a motor-driven glass homogenizer. This time was selected because no differences were found in the respiratory rates of tissues homogenized for one, three, or five minutes.

[1] This investigation was supported in part by the Medical Research and Development Board, Office of the Surgeon General, Department of the Army, under Contract No. DA-49-007-MD-444.

Oxygen consumption was measured in Warburg constant volume manometers according to the procedure outlined by Umbreit, Burris and Stauffer (1945). The center well of the Warburg flask contained 0.2 ml. of a 10 per cent KOH solution to which was added a piece of folded filter paper to increase the surface of the KOH

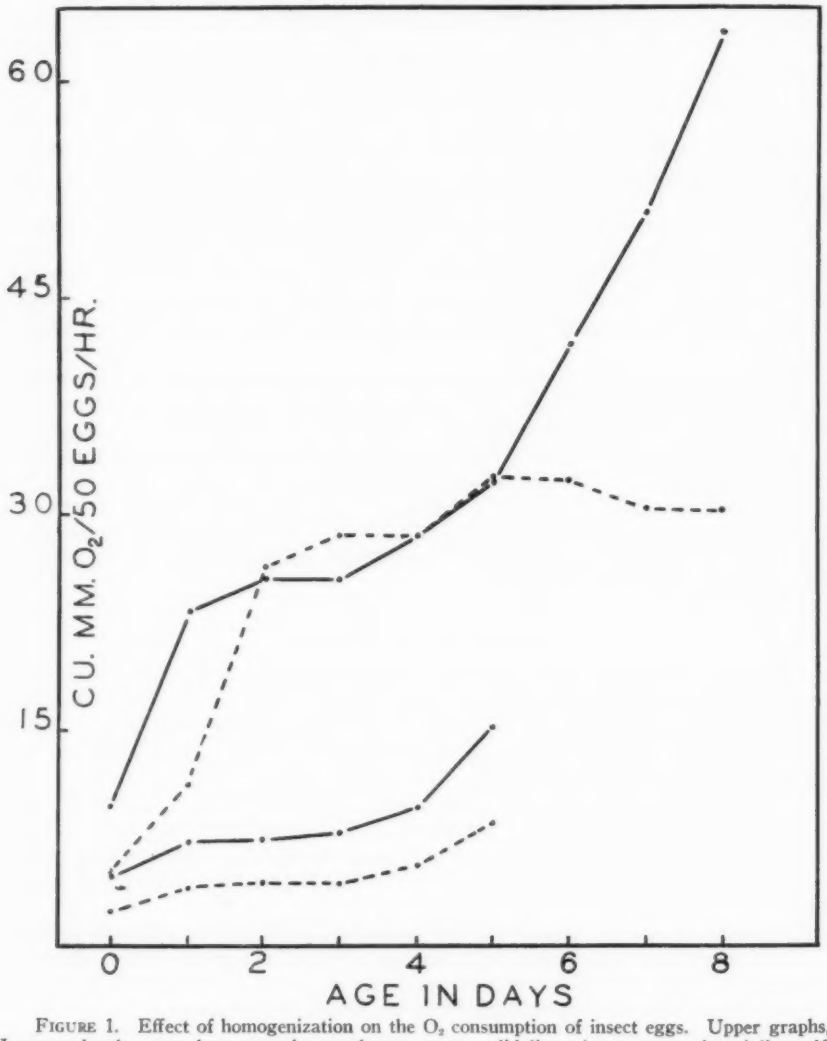

FIGURE 1. Effect of homogenization on the O_2 consumption of insect eggs. Upper graphs, Japanese beetle eggs; lower graphs, mealworm eggs; solid lines, intact eggs; dotted lines, 10 per cent homogenates. Graph for intact Japanese beetle eggs taken from Ludwig and Wugmeister (1955).

TABLE I

Oxygen consumption of whole mealworm larvae and of homogenates of mealworm larvae

Per cent homogenate	No. of readings	Oxygen consumption μ liters/gram/minute (with standard errors)	Per cent decrease
Whole larvae	46	10.17 ± 0.73	
20	18	7.25 ± 0.28	28.7
10	71	6.62 ± 0.34	34.9
7.5	18	7.45 ± 0.73	26.7
5	42	6.06 ± 0.33	40.4
2.5	18	5.46 ± 0.58	46.3
1	49	3.65 ± 0.34	64.1

solution. The manometers were placed at a temperature of $30° \pm 0.01°$ C. Readings were made at 15-minute intervals for a period of two hours. During this time the manometers were rocked horizontally 100 times per minute.

OBSERVATIONS

A comparison of the rates of oxygen consumption of whole Japanese beetle eggs and of their homogenates for each day of embryonic development is shown in Figure 1. Each point in this figure represents an average of at least 10 determinations. Respiration of 10 per cent homogenates, expressed as μ liters of oxygen/50 eggs/hour, was 5.1 in newly laid eggs. It increased to 26.2 during the next two days and remained relatively constant at approximately 30 during the remainder of the embryonic period. A comparison of these results with those of Ludwig and Wugmeister (1955) shows that homogenizing the newly laid egg reduced oxygen consumption by approximately 50 per cent. It did not change the respiratory rate from the third to the sixth days of incubation, but towards the end of the embryonic period, it again resulted in a reduction of more than 50 per cent. On the other hand, the amount of reduction was maintained constant throughout the embryonic period in the mealworm. Using 10 per cent homogenates, it was found that homogenizing the egg of this species resulted in a reduction of approximately 48 per cent in the rate of oxygen consumption (Fig. 1).

The rates of oxygen consumption of whole mealworm larvae and of their homogenates are listed in Table I. Oxygen consumption of whole larvae averaged 10.17 μ liters/gram/minute. That of homogenates depended upon concentration, being 7.25 in 20 per cent, and only 3.65 in 1 per cent homogenates. In the former case, the reduction amounted to approximately 29 per cent; and in the latter, to 64 per cent.

The effects of homogenization on the rate of oxygen consumption of the mealworm during metamorphosis are shown in Figure 2. Each point in Graph A represents an average of at least 10, in Graph B of 30, and in Graph C of 12 determinations. The oxygen consumption of whole insects followed the characteristic U-shaped curve first described for this species by Krogh (1914). It was high immediately after pupation, decreased to a minimum in the two-day pupa, and then increased during the remainder of the pupal stage. The oxygen uptake of 1 per cent

and of 5 per cent homogenates remained relatively constant during the pupal stage, but increased in newly emerged adults. Hence, the amount of reduction resulting from homogenization varied with the stage of metamorphosis. With 1 per cent homogenates, it was approximately 61 per cent in prepupae, 80 per cent in newly molted, 50 per cent in two-day, 72 per cent in five-day pupae, and 80 per cent in newly emerged adults. The oxygen uptake of 5 per cent homogenates was considerably higher than that of the 1 per cent. However, its rate was also relatively constant throughout the pupal stage.

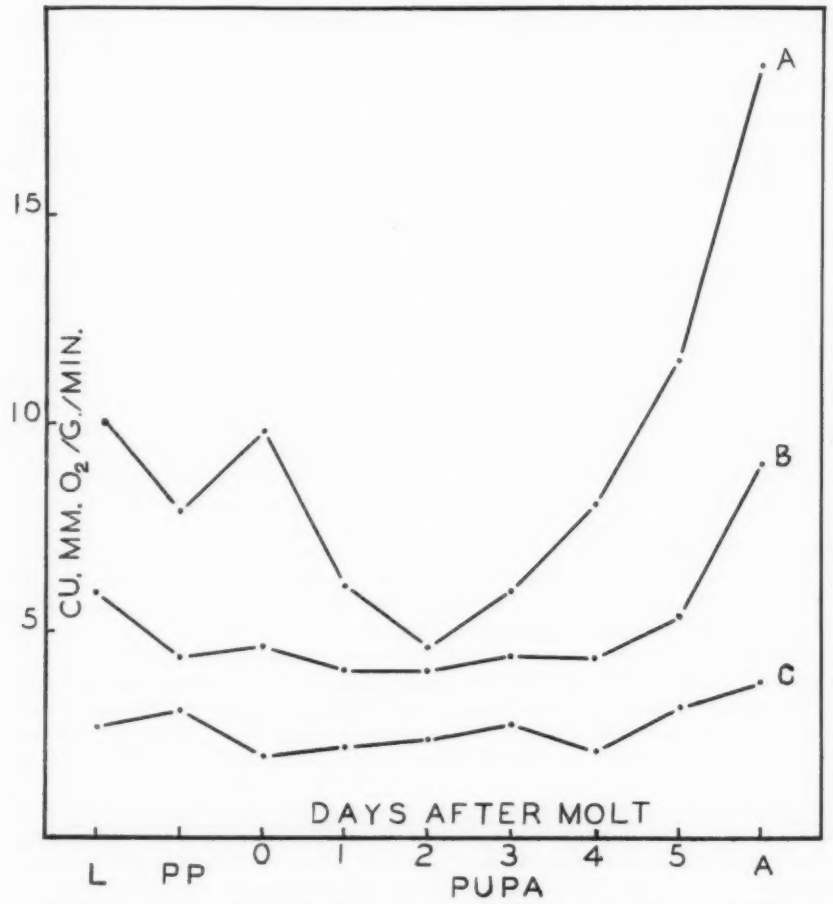

FIGURE 2. Effect of homogenization on the O_2 consumption of mealworms during metamorphosis. Graph A, intact insects; graph B, 5 per cent homogenates; graph C, 1 per cent homogenates; L, larvae; PP, prepupae; A, newly emerged adults.

Discussion

The varying effects of homogenization on oxygen consumption throughout the embryonic period of the Japanese beetle may possibly be correlated with water content and degree of organization of the egg. Rothstein (1952) reported that the newly laid egg weighed only 0.83 mg. and contained 50 per cent water. Hence, the various constituents are in a rather concentrated condition. During the first four days of development at 30° C., the egg increased in weight to 2.3 mg. and its water content increased to more than 80 per cent. Hence, approximately 1.5 mg. of water were imbibed by each egg and the enzymes and other constituents were greatly diluted. At this time homogenization appears to have no effect on the rate of oxygen consumption. Gese (1953) found that blastokinesis occurs on the fifth day at 30° C., followed by the differentiation of the embryo. This period of differentiation was shown by Ludwig and Wugmeister (1955) to be associated with a rapid increase in respiratory rate and an increase in the activities of cytochrome oxidase and succinic dehydrogenase. Homogenization of the egg during this period of differentiation resulted in a marked reduction in oxygen consumption, probably due to the disruption of organization in the egg. On the other hand, mealworm eggs do not imbibe water but maintain a constant weight throughout the embryonic period. Homogenization of these eggs resulted in a constant reduction of approximately 48 per cent in oxygen consumption. The difference between the present results with mealworm eggs, and those of Bodine and Lu (1951) with grasshopper eggs (48 as compared to 65 per cent), may be associated with homogenate concentration. Furthermore, Bodine and Lu used embryos only, while in the present experiments whole eggs were used.

The elimination of the U-shaped respiratory curve in the pupal stage of an insect by homogenization was also reported by Cotty (1955) with the house fly, *Musca domestica*. These observations add evidence for the belief that the U-shaped curve is associated with different degrees of tissue organization (Krogh, 1914; Fink, 1925). When this organization is disrupted by homogenization, a uniform rate is produced. However, homogenization may also affect respiratory enzymes and thus modify the rate of oxygen consumption as indicated by the work of Handler and Klein (1942) who showed that DPN is rapidly broken down by tissue homogenates thus blocking various dehydrogenases.

The present experiments indicate that the effects of homogenization on the respiratory rate of insect tissues vary with the stage of development, physiological differences among various species, and homogenate concentration.

Summary

1. The oxygen consumption of 10 per cent homogenates of Japanese beetle and mealworm eggs was measured for each day of the embryonic period at 30° C. and compared with that of intact eggs.

2. Homogenization of newly laid Japanese beetle eggs resulted in a reduction of 50 per cent in oxygen consumption. From the third to sixth day, there was no decrease but towards the end of the embryonic period, a reduction of more than 50 per cent was again obtained. Homogenization of mealworm eggs resulted in an average reduction of 48 per cent in oxygen consumption which remained constant throughout the embryonic period.

3. The rates of oxygen consumption of whole mealworm larvae and of their homogenates were determined. Homogenization resulted in a decrease in respiratory rate, the extent depending on homogenate concentration. With 20 per cent homogenates, there was a reduction of 29 per cent; and with 1 per cent homogenates, it was 64 per cent.

4. The effects of homogenization on the respiratory rate of mealworms were determined for each day of metamorphosis at 30° C. Homogenization abolished the U-shaped respiratory curve characteristic of intact pupae. Therefore, the degree of reduction depended on the stage of metamorphosis. With 1 per cent homogenates, it was 61 per cent in prepupae, 80 per cent in newly molted, 50 per cent in two-day, 72 per cent in five-day pupae, and 80 per cent in newly emerged adults. Furthermore, the extent of reduction also varied with homogenate concentration being greater in 1 per cent than in 5 per cent homogenates.

LITERATURE CITED

Bodine, J. H., and K. H. Lu, 1951. Structure and endogenous oxygen uptake of embryonic cells. *Physiol. Zool.*, **24**: 120–126.

Cotty, V. F., 1955. Respiratory metabolism of prepupae and pupae of the house fly, *Musca domestica* L., and of their homogenates. Ph.D. thesis, New York University.

DeRobertis, E. D. P., W. W. Nowinski and J. F. Saez, 1950. General cytology. W. B. Saunders Co., Philadelphia.

Fink, D. E., 1925. Metabolism during embryonic and metamorphic development of insects. *J. Gen. Physiol.*, **7**: 527–543.

Gese, E. C., 1953. Embryogenesis of the Japanese beetle, *Popillia japonica* Newman, and a correlation of morphological events with known physiological changes. Ph.D. thesis, New York University.

Handler, P., and J. R. Klein, 1942. The inactivation of pyridine nucleotides by animal tissues *in vitro*. *J. Biol. Chem.*, **143**: 49–57.

Krogh, A., 1914. On the rate of development and CO_2 production of chrysalides of *Tenebrio molitor* at different temperatures. *Zeitschr. f. allg. Physiol.*, **16**: 178–190.

Ludwig, D., and M. Wugmeister, 1955. Respiratory metabolism and the activities of cytochrome oxidase and succinic dehydrogenase during the embryonic development of the Japanese beetle, *Popillia japonica* Newman. *J. Cell. Comp. Physiol.*, **45**: 157–165.

Rothstein, F., 1952. Biochemical changes during the embryonic development of the Japanese beetle (*Popillia japonica* Newman). *Physiol. Zool.*, **25**: 171–178.

Umbreit, W. W., R. H. Burris and J. F. Stauffer, 1945. Manometric techniques and related methods for the study of tissue metabolism. Burgess Publishing Co., Minneapolis.

RECIPROCAL PROTOZOAN TRANSFAUNATIONS BETWEEN THE ROACH, CRYPTOCERCUS, AND THE TERMITE, ZOOTERMOPSIS[1]

W. L. NUTTING[2]

Department of Biology, Harvard University, Cambridge, Mass.

A technique has recently been described whereby the symbiotic protozoa of the wood-feeding roach, *Cryptocercus,* may be transferred from the hind-gut of one host to another *per rectum* (Cleveland and Nutting, *J. Exp. Zool.,* in press). The method has now been applied successfully in the reciprocal transfer of protozoa between this roach and the large, western North American termite, *Zootermopsis.*

An earlier series of experiments by Cleveland, in which the transfer was accomplished by feeding, showed that the protozoa were capable of living in alternate hosts for some time (Cleveland *et al.,* 1934, p. 320). This preliminary work called for more extensive experiments, first to determine whether the termite protozoa could indeed supplant the normal infection of the roach and vice versa. Should this then be the case, it would be a matter of considerable interest to determine the behavior of the protozoa in their new hosts during the molting period.

Experimentation of this sort has been extremely limited and largely confined to determining the host specificity of protozoa from certain termites. Light and Sanford (1928) reinfected oxygen-defaunated termites *per rectum* with a glass canula connected by rubber tubing to a mouthpiece. They found that the protozoa of *Zootermopsis angusticollis* could live and multiply in *Kalotermes hubbardi* for at least 100 days. Dropkin (1941) similarly infected *Z. angusticollis* with protozoa from *Reticulitermes flavipes,* and *Kalotermes jouteli* and *schwarzi.* None of the protozoa multiplied, and so were unable to support the new host for longer than 50 days. He therefore concluded that these unsuccessful refaunations resulted from physiological as well as morphological distinctions which must exist between the protozoa from widely different species of termites.

Before describing the roach-termite transfaunations, it is pertinent to recount the natural methods for transfer of protozoa between the normal hosts. *Cryptocercus* harbors a large and diverse fauna composed of 13 described genera and some 30 species of hypermastigote and polymastigote flagellates. All of these genera change from asexual to sexual methods of reproduction during the molting period of their host which, in the laboratory at 21° C., lasts for about 44 days. Most of the individuals of *Trichonympha* encyst a few days before molting, and large numbers are usually passed in the fecal pellets within 20 to 36 hours after ecdysis. The hatching of nymphs from their oothecae coincides with the peak of the molting season, so that the new individuals are able to gain their initial infections, at least in

[1] This work was supported in part by a grant from the National Science Foundation and by grant E-853 from the Microbiological Institute, National Institutes of Health, U. S. Public Health Service.

[2] Present address: Department of Entomology, University of Arizona, Tucson.

part, by feeding on these pellets. This infection is never lost naturally, even during succeeding ecdyses. Adults and intermolt nymphs do not normally pass protozoa in their fecal pellets, except for an occasional small polymastigote (Cleveland *et al.*, 1934). From observations made on incipient colonies in nature, it is becoming evident that there must be some other means of transferring these protozoa from intermolt nymphs, and probably adults, to the newly hatched nymphs. Repeated attempts to discover such a method in the laboratory have thus far been unsuccessful.

On the other hand, the five genera and seven species of flagellates symbiotic in the hind-gut of *Zootermopsis* do not undergo any sexual cycles, and die shortly before each ecdysis. Occasionally, a few individuals of *Tricercomitus* and/or *Streblomastix* remain in the hind-gut through this period. These recently molted and defaunated termites regain their infections by the solicitation of proctodaeal "food," which contains active protozoa, from their non-molting associates.

Materials and Methods

Specimens of *Cryptocercus punctulatus* Scudder have been collected annually in the vicinity of the Mountain Lake Biological Station of the University of Virginia and maintained in Cambridge for a number of years. Cultures of *Zootermopsis angusticollis* (Hagen) were collected in the vicinity of Placerville, Eldorado County, California, and additional lots from Pacific Grove were kindly supplied by Miss Frances M. Weesner of the University of California, Berkeley. Groups of experimental animals were maintained at 20° C. in petri dishes, supplied with wood and damp paper toweling, and cleaned frequently. This is perhaps not the best method for keeping termites in the laboratory, but it has proven very satisfactory where frequent examination is of first importance.

In brief, the transfaunation technique involves the transfer, *per rectum*, of a fresh inoculum of protozoa to a defaunated recipient. The recipients, either roaches or termites, are previously defaunated under 45 lb. of O_2 for 1.5 hours (Cleveland, 1925a), and later relaxed under CO_2 for the actual transfer. Just prior to the operation the hind-gut contents of the roaches are squeezed out by gentle manipulation of the abdomen. While struggling under CO_2, termites conveniently expel their hind-gut contents almost completely. The inoculum, obtained from the hind-gut of one or more freshly killed donors, is drawn up into a length of polyethylene tubing attached to a syringe. The tube is then guided between the paraprocts into the rectum where the inoculum is released. It is thus possible to replace the original population of a termite with an equivalent volume of protozoa from a roach and vice versa.

Experimental

1. *Transfer of termite protozoa to roaches*

The natural fauna of nine adult and nine nymphal *Cryptocercus* was replaced with protozoa from *Zootermopsis*. In order to examine the entire gut contents and determine the progress of the infection, three adults were killed between 8 and 24 days. In the first, *Trichomonas termopsidis* and *Trichonympha sphaerica* were much more abundant in the anterior than in the posterior colon. The second con-

tained only *Trichomonas,* but in fair numbers. The third contained all forms in normal numbers. Three more were killed for examination after 72 days. These harbored normal numbers of protozoa, but the populations were composed of more *Trichomonas* and fewer *Trichonympha* than are usually found in the termite host.

The remaining adults were examined shortly before presumably normal deaths after 192, 207, and 245 days, for these animals had then been kept in the laboratory for about one year. The first two contained vigorous infections composed of all forms in their usual proportions. Only *Trichomonas* and *Tricercomitus termopsidis* remained in fair abundance in the last adult. It seems probable that other forms were previously present, but died because of the moribund condition of the host.

Of the nine transfaunated *Cryptocercus* nymphs, inoculated with *Zootermopsis* protozoa, three were killed at or near death after 49, 68, and 81 days. One contained a few *Trichomonas* only; the second a few *Trichomonas* and *Trichonympha;* and the third was completely defaunated. Three more were killed after 61, 78, and 83 days, and all had excellent infections. *Trichomonas* was absent from one of these animals, although it is normally very abundant. A sample of protozoa was squeezed from the hind-gut of another nymph after 221 days. All genera and species were present and as abundant and active as though they had just been taken from their original host. Unfortunately, this animal escaped a few days later.

Of the remaining two nymphs, one was examined after 97 days and about one day before ecdysis. *Tricercomitus* and *Streblomastix* were active and present in normal numbers. A few individuals each of *Trichomonas* and *Trichonympha* were behaving normally, but the remainder were either non-motile or already degenerating. The other was examined after 82 days and about 12 hours before ecdysis. *Tricercomitus* was again present in normal numbers, and one *Streblomastix* was seen. No other protozoa were present, but a few unidentifiable remains of larger forms still persisted.

2. *Transfer of roach protozoa to termites*

Fifty-five individuals of *Zootermopsis angusticollis* were defaunated and reinfected with *Cryptocercus* protozoa. These included approximately equal numbers of first, second, and third form reproductives, averaging about 1 cm. long and at least two instars from maturity. Twelve individuals were killed to check the progress of the new infections during the first 50 days. Seven of these had become rather feeble and contained small populations of *Hexamita cryptocerci* and one or two other small polymastigotes, or else were completely defaunated. The remainder contained vigorous, normally proportioned infections. These incomplete faunas, both in transfaunated roaches and termites, are almost certainly due to inadequate refaunation. The early deaths probably resulted from starvation following these poor infections, or from gut injuries incurred during the transfer. Over a period of about one year some 20% of the animals have died in dry or moldy cultures, or have been eaten by other members of the colony for one reason or another. This is not a high mortality figure for these insects maintained in small numbers in such loosely covered containers.

Eight more were examined at ecdysis and at intervals up to 24 hours later.

These were completely defaunated or nearly so. Three individuals contained dead and dying *Trichonympha, Barbulanympha,* and *Saccinobaculus,* along with abundant remains of other large *Cryptocercus* protozoa. A few of them contained insignificant numbers of one or more of the following: *Hexamita* and other very small polymastigotes (from *Cryptocercus*); *Tricercomitus, Trichomonas,* and *Streblomastix* (from *Zootermopsis*). An occasional individual has since been found still harboring a small number of the last three genera, showing that they were not always completely removed by the defaunation time employed here. The three species of *Trichonympha,* dominant forms in the termite, have never been found to survive this treatment.

It should be stated parenthetically that *Streblomastix* is not a symbiont, and is dependent either upon its host or, more probably, upon the other protozoa for its nourishment. *Trichomonas* is symbiotic, but it is doubtful that it alone could maintain its host indefinitely (Cleveland, 1925b). Although the relationships between *Tricercomitus* and *Hexamita* and their hosts have not been studied, it is questionable whether they are truly symbiotic, and if so, whether they could furnish adequate support indefinitely.

Six termites were judged to be approaching ecdysis by reason of their distended appearance and internally divided third and fourth antennal joints. Periodic samples of protozoa were squeezed from these insects and definite evidence of sexual cycles was found in several genera. Several stages of fertilization in *Saccinobaculus* were most commonly seen. These stages ranged from forms where gametes had recently fused, to those in which axostylar fusion was completed and the pronuclei had become juxtaposed (Cleveland, 1950b). Stages of fertilization in *Oxymonas* and *Notila* were also seen in one termite (Cleveland, 1950a, 1950c). *Barbulanympha,* with two nuclei and two flagellated areas (probably gametogenesis), was observed in another (Cleveland, 1953).

In several termites, *Barbulanympha, Trichonympha,* and even the sexually active polymastigotes, were dying or degenerating. One of these termites, which contained *Saccinobaculus* with fusing pronuclei, molted 17 days later and was completely defaunated. The remaining individuals were apparently unable to survive the frequent examinations.

Recently molted termites have repeatedly regained their infections of *Cryptocercus* protozoa from other members of the colony just as they do their own protozoa. Moderate numbers of *Hexamita* and other small polymastigotes become established within 3 or 4 days after molting and adequate refaunations of all protozoa are attained within 15 days, or perhaps less.

Still more convincing evidence in the form of laboratory-established colonies is accumulating to show that *Cryptocercus* protozoa can probably support *Zootermopsis* indefinitely. One third form reproductive (with no indication of wing pads), isolated with five others, began founding an incipient colony in January, 1956. All six of these termites had been infected with roach protozoa seven months previously. Four months later some two dozen nymphs, representing at least three instars, had been produced by this queen. Several of these nymphs were examined and found to contain well balanced faunas of roach protozoa. A second form reproductive (with short wing pads), accompanied by two third form reproductives similarly infected, also began founding a new colony at about the same time.

Within the same period a half-dozen nymphs had been produced. Examination of one of these showed a good infection of *Cryptocercus* protozoa.

3. Effects of reduced oxygen tension

The possibility has been raised frequently that the protozoan sexual cycles might be caused by certain changes among the physical factors within the host's hind-gut —most notably a reduction of the oxygen tension. The following experiments, although complete in themselves, more properly form part of a separate and more extensive study of such factors. Their pertinence, however, requires that they be described briefly at this time. Two series of *Zootermopsis* were subjected to varying mixtures of O_2 and N_2 (40% O_2–60% N_2 to 0.5% O_2–99.5% N_2) at atmospheric pressure. In one series the termites contained their own protozoa; in the second, *Cryptocercus* protozoa. It was found that these termites could live in an atmosphere of 2% O_2 and 98% N_2, probably indefinitely. They survived and molted normally under these conditions for about two months. In an atmosphere of 0.5% O_2 and 99.5% N_2 they are nearly defaunated in 24 hours; *Trichomonas* and *Tricercomitus* might survive somewhat longer. The termites themselves were practically immobile during this time, but still recovered when returned to a normal atmosphere. In an atmosphere of 1.0% O_2 the termites survive rather lethargically for two weeks or somewhat longer, but are slowly and irregularly defaunated. Therefore, with the host in the narrow range between 1 and 2% O_2, the protozoa are the first of the partners in the symbiosis to succumb.

Similar experiments, with *Cryptocercus* containing *Zootermopsis* protozoa, have not been carried out although normally faunated roaches are defaunated without injury in two days in an atmosphere of 1% O_2 and 99% N_2. The roaches themselves are unable to recover after three days under these conditions.

The significant point here is that, even under these conditions, the protozoan behavior within the termite is identical to that already observed under normal atmospheric conditions. That is, the termite protozoa continue their usual preecdysial degeneration; not a single organism was seen which could have been considered in the process of sexual reproduction. The roach protozoa continue the initiation of sexual cycles which abort shortly before ecdysis. Reduced oxygen tension by itself, therefore, does not cause the initiation of sexual cycles in either termite or roach protozoa in the termite host. Neither does it assist or hinder the established behavior of the roach protozoa in the termite host. A more thorough discussion on the control of the sexual cycles of the roach protozoa is presented in a separate paper (Cleveland and Nutting, *J. Exp. Zool.*, in press).

Finally, brief mention should be made of the rod-like bacteria (the so-called cuticular striations) regularly found embedded in the pellicle of *Urinympha* and *Barbulanympha*. It is not known whether these microorganisms subserve any nutritive or other function to either protozoa or roach (Cleveland, 1951). They are not normally found on any other protozoan genera in roaches from the Appalachian region, nor on any of the protozoa of *Zootermopsis*. *Urinympha* and *Barbulanympha* retain their embedded bacteria when transferred to the termite, but, what is more interesting, the same or very similar bacteria sometimes become established on the large, protean polymastigote, *Saccinobaculus*, whenever it is so transferred.

Discussion

These experiments demonstrate conclusively that the protozoan complement of *Cryptocercus* can maintain the termite, *Zootermopsis*, through significant portions of two generations and probably indefinitely. They also prove that *Zootermopsis* protozoa can adequately maintain nymphal *Cryptocercus* as long as they do not molt, and mature roaches probably for the normal span of adult life.

The death of the roach protozoa at each molt presents the termite with no more of a problem than does the natural loss of its own protozoa at this time. Aside from the requisite competence of the protozoa themselves, the continuity of this unnatural symbiosis is made possible through the direct method of refaunation, *i.e.*, the solicitation of proctodaeal fluid, already employed by the termites.

On the other hand, the gradual and unnatural loss of termite protozoa during the molting period presents the nymphal roach with an apparently insurmountable problem, and it then dies of starvation. These termite protozoa are as incapable of surviving the molting environment of the roach hind-gut as they are that of their normal host. Earlier experimentation showed that reinfection by the solicitation of proctodaeal food is probably a behavioral impossibility for the roach (Cleveland *et al.*, 1934), although this matter demands further investigation.

Recent work supports the thesis that hormonal mechanisms responsible for growth and molting may also regulate the protozoan sexual cycles in *Cryptocercus* (Cleveland and Nutting, *J. Exp. Zool.*, in press). This work has shown that the synchronization between protozoa and developing host must be very precise in order for the protozoa to accomplish their cycles. The present finding that the roach protozoa actually begin sexual cycles in molting period termites provides encouraging support. The fact that the cycles abort, with resulting degeneration of the protozoa, parallels precisely the behavior of sexual protozoa after they are transferred to another host roach which is more or less advanced in the molting period. It is, therefore, hardly surprising that both roach and termite protozoa are unable to adapt completely to the hormonal milieu of such divergent hosts.

There is much evidence to support the belief that the termites, together with their symbiotic protozoa, are derived from a line of xylophagous roaches. In areas where their ranges are coincident today, *Zootermopsis* and *Cryptocercus* may be found in contiguous galleries within the same log. This fact, together with the present finding that the protozoa of these insects are reciprocally transferable, adds considerable support to the derivation of termites from a line of roaches much like *Cryptocercus* and perhaps even from the same stock.

Finally, a most intriguing observation deserves mention at this time, for it is strongly suggestive of a radical change in the hormonal milieu of the termite host at the time of the imaginal molt. Last stage reproductives are *not* completely defaunated at this time as is the rule with all forms at each preceding molt. This has been determined by the examination of pre-adult reproductives from a few days before, and until, ecdysis. There is much less wood in the hind-gut of these forms and the total number of protozoa is diminished. The general behavior of these protozoa as observed in isolated reproductives at ecdysis and shortly thereafter, has shown that their survival is assured, however.

Pflugfelder (1947) has reported that the corpora allata increase in size in reproductive nymphs of *Microcerotermes amboinensis* and continue to enlarge for a

time after the imaginal molt. This is not the case with soldiers and the so-called workers. Furthermore, in the sexual forms the growth of the ventral glands parallels that of the corpora allata until the final molt, at which time these glands degenerate. The corpora allata and ventral glands do not enlarge nor do the ventral glands degenerate, in the soldiers and workers. He considered the hypertrophy of the allata, at least, to be concerned with sexual metabolism.

The successful establishment of new colonies demands that each founding pair be provided with protozoa. It is quite possible that there may be neither time nor opportunity for these forms to obtain their infections in the usual manner after the final molt. Enormous numbers of these winged forms mature almost simultaneously, and the nuptial flight generally occurs within a short time thereafter. The observations presented in this paper, coupled with the findings of Pflugfelder, suggest that these obvious glandular changes, and the resulting shifts in hormone balance may constitute a mechanism for insuring the adult termites a supply of protozoa for their nuptial flight.

Summary and Conclusions

In the wood-feeding roach, *Cryptocercus,* most members of the 13 genera of intestinal flagellates change from asexual to sexual methods of reproduction each time their host molts. Members of one genus encyst some time before, and pass out with fecal pellets shortly after ecdysis. Since hatching is synchronized with molting, the young nymphs are thus enabled to acquire at least a part of their initial infections, which are then retained for life. The five flagellate genera found in the termite *Zootermopsis* undergo no sexual cycles and, with few exceptions, they all degenerate shortly before the molt. The termites therefore must refaunate themselves after each molt, and this they do by the solicitation of proctodaeal droplets containing protozoa from their non-molting associates. All protozoa may be removed from the roach and the termite with oxygen and without injury to either host. Reciprocal transfers of normal protozoan faunas have been made *per rectum* between these two insects.

The termite protozoa can live in and adequately maintain nymphal roaches for as long as 221 days, or probably as long as they do not molt. The protozoa can maintain adult roaches for at least as long as 245 days, or probably for the duration of adult life. However, examination of nymphal roaches one day or less before the molt showed that the vital majority of the protozoa became feeble, non-motile, and finally degenerated before this event took place. No evidence of sexual cycles was observed. These defaunated nymphs are behaviorally incapable of regaining an infection of termite protozoa under natural conditions and must shortly die of starvation. Thus, except under experimental conditions, termite protozoa could not indefinitely maintain a colony of *Cryptocercus.*

The roach protozoa, on the other hand, actually have supported individual termites for as long as one year, and through several instars. Some genera of the protozoa started sexual cycles in molting period termites, but these were all aborted and the termites were practically defaunated at ecdysis. But since they are naturally defaunated at this time, they were also able to regain infections of roach protozoa from other individuals so infected. In addition, second and third form reproductives containing roach protozoa have established small colonies in the

laboratory, and have successfully reared nymphs through at least four instars. *Cryptocercus* protozoa are therefore very likely capable of supporting a colony of this termite indefinitely.

Various implications of these results are discussed, especially with regard to possibilities of determining the role of the host in initiating and controlling the sexual cycles of *Cryptocercus* protozoa.

LITERATURE CITED

CLEVELAND, L. R., 1925a. The effects of oxygenation and starvation on the symbiosis between the termite, *Termopsis*, and its intestinal flagellates. *Biol. Bull.*, **48**: 309–327.

CLEVELAND, L. R., 1925b. Toxicity of oxygen for protozoa in vivo and in vitro: animals defaunated without injury. *Biol. Bull.*, **48**: 455–468.

CLEVELAND, L. R., 1950a. Hormone-induced sexual cycles of flagellates. II. Gametogenesis, fertilization, and one-division meiosis in Oxymonas. *J. Morph.*, **86**: 185–214.

CLEVELAND, L. R., 1950b. Hormone-induced sexual cycles of flagellates. III. Gametogenesis, fertilization, and one-division meiosis in Saccinobaculus. *J. Morph.*, **86**: 215–228.

CLEVELAND, L. R., 1950c. Hormone-induced sexual cycles of flagellates. IV. Meiosis after syngamy and before nuclear fusion in Notila. *J. Morph.*, **87**: 317–348.

CLEVELAND, L. R., 1951. Hormone-induced sexual cycles of flagellates. VII. One-division meiosis and autogamy without cell division in Urinympha. *J. Morph.*, **88**: 385–440.

CLEVELAND, L. R., 1953. Hormone-induced sexual cycles of flagellates. IX. Haploid gametogenesis and fertilization in Barbulanympha. *J. Morph.*, **93**: 371–404.

CLEVELAND, L. R., S. R. HALL, E. P. SANDERS AND J. COLLIER, 1934. The wood-feeding roach *Cryptocercus*, its protozoa and the symbiosis between protozoa and roach. *Mem. Amer. Acad. Arts and Sci.*, **17**: 185–342.

CLEVELAND, L. R., AND W. L. NUTTING. Suppression of sexual cycles and death of the protozoa of *Cryptocercus* resulting from change of hosts during molting period. *J. Exp. Zool.*, in press.

DROPKIN, V. H., 1941. Host specificity relations of termite protozoa. *Ecology*, **22**: 200–202.

LIGHT, S. F., AND M. F. SANFORD, 1928. Experimental transfaunation of termites. *Univ. Calif. Publ. Zool.*, **31**: 269–274.

PFLUGFELDER, O., 1947. Über die Ventraldruesen und einige andere inkretorische Organe des Insektenkopfes. *Biol. Zentralbl.*, **66**: 211–235.

ON THE RELATION BETWEEN GROWTH AND MORPHOGENESIS IN THE SLIME MOLD DICTYOSTELIUM DISCOIDEUM [1]

MAURICE SUSSMAN

Dept. of Biological Sciences, Northwestern University, Evanston, Illinois

In the normal course of cellular slime mold development, the myxamoebae pass through the exponential growth phase and, upon its cessation, aggregate in multicellular masses. The number of aggregative centers formed has been shown to be a function of the number of cells and of the population density (Sussman and Noel, 1952). From these and other relationships (Sussman, 1952) it can be inferred that each aggregate requires for its formation the presence of a special cell called an initiator, to which neighboring cells respond, and only a small proportion of the population can so serve under the conditions employed. In *Dictyostelium discoideum* wild type, the ratio of the initiator to responder cells has been found to be 1:2200. That the system is under genetic control is shown by the fact that mutants of *D. discoideum,* as well as wild type representatives of other species, display significantly different ratios (Sussman and Noel, 1952; Sussman, 1955).

In the past, Raper (1940) has presented evidence to indicate that aggregation can begin only when growth ceases and that the aggregation of a stationary phase population can be prevented and indeed reversed by further additions of nutrient material. The present study demonstrates an extension of this conclusion: that is, that cells taken from any phase of the growth cycle, including newly germinated spores, can aggregate when further growth is prevented and, in fact, give quantitatively the same aggregative performance as do stationary phase myxamoebae.

Methods

A. Organisms and media. The organism used in this study is representative of *D. discoideum* Raper, strain NC-4. Stock cultures were grown on glucose-peptone agar in association with *A. aerogenes* by methods previously described (Sussman, 1951). Under these conditions the myxamoebae exhaust the bacterial supply after about 44 hours incubation at 22° C. and aggregate and construct fruits in the ensuing 24 hours. Spore suspensions from these fruits were used as inocula for special growth experiments to be described in the next section.

B. Aggregation experiments. All the methods and procedures employed have been described in detail elsewhere (Sussman and Noel, 1952). Washed myxamoebae were suspended in salt solution (Bonner, 1947), counted with a hemocytometer, and dispensed in drops on a washed agar-distilled water substratum. The excess fluid was absorbed by the agar leaving the cells randomly distributed within the confines of the drop. Under these conditions the amoebae remained constant in number, aggregated in normal fashion after 12 hours and produced

[1] This study was aided by grants from the Office of Naval Research and The National Institutes of Health, U.S.PH.S.

typical fruits. Control of the number of myxamoebae delivered was achieved by appropriate dilution of the original cell suspensions. The population density of the cells on the agar was determined by the volume of the drop delivered.

Results

A. The growth curve of D. discoideum on complete and restricted media

Spores, taken from a 4–6 day stock plate, were suspended in salt solution and spread on glucose peptone agar with 0.1 cc. of a 48-hour broth culture of *A. aerogenes*. The initial spore count per plate was 1×10^6. At intervals, duplicate plates were harvested using a finger and 10 cc. distilled water delivered in two batches. Control experiments using known numbers of cells indicated a recovery in excess of 95% of the cells on the plates. Replicate counts with the hemocytometer were made of each harvest. Figure 1 shows the results of two experiments (upper curve).

A technical difficulty precluded the use of this system for studying the aggrega-

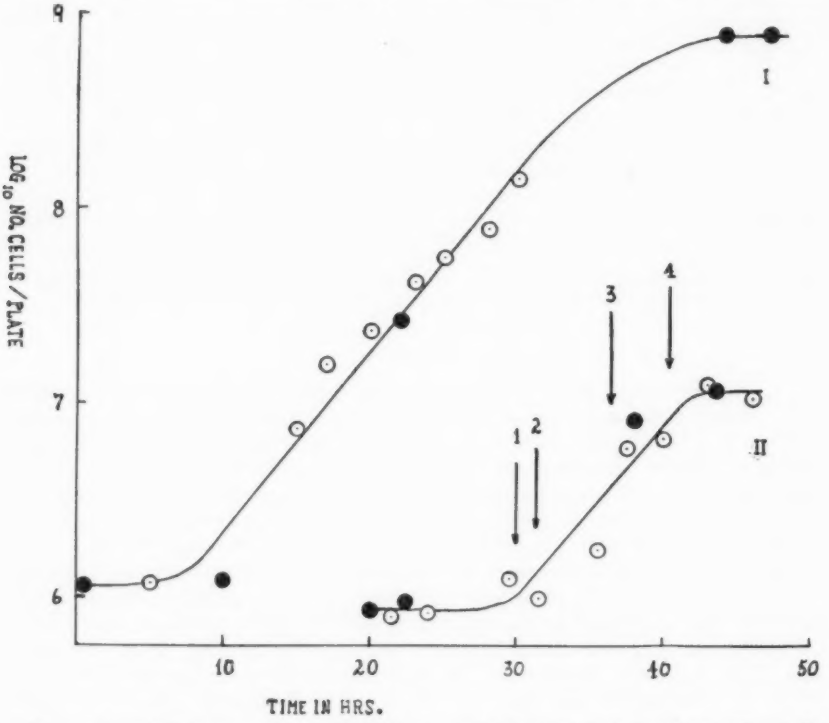

Figure 1. Growth curves of *D. discoideum* on complete (I) and restricted (II) media. The arrows indicate the times at which cell samples were taken for tests of aggregative performance.

tive performances of cells in different growth phases. During the log phase the number of bacteria was so great that clean preparations could not be obtained even by exhaustive washing in the centrifuge. The delivery of such contaminated suspensions to washed agar plates for the aggregation experiments led to considerable growth of the myxamoebae and rendered the results meaningless.

To avoid this hindrance, a restricted medium was used on which the bacteria remained in more convenient balance with the myxamoebae. A 48-hour broth culture of *A. aerogenes* was washed once in the centrifuge and suspended in water to ½ the original volume. One-half cc. aliquots were spread on washed agar plates with the spore inoculum and incubated at 22° C. Figure 1 (lower curve) shows the growth curve obtained from two experiments. The arrows show where samples of cells were taken for aggregation experiments to be described. The data reveal only minor differences in the growth curves on the two types of media. The lag phase, including the germination period, was about 7 hours on complete medium and about 11 hours on restricted. True exponential increases occurred on both media, the generation times being approximately the same (ca. three hours).

The stationary phase was attained at about 40 hours on complete medium and at 24 hours on restricted medium. A population of nearly 10^9 myxamoebae per plate on the former indicated a total of 9.5 generations of growth. On the latter medium the stationary population was slightly more than 10^7 per plate, the result of 3.8 generations of growth.

B. Aggregative performance of cells in different growth phases

As mentioned previously the number of centers of aggregation formed by a population of myxamoebae is a function of the number of cells and of the population density. The curve in Figure 2 demonstrates these relationships for samples of 10,000 washed myxamoebae taken from the stationary growth phase on glucose-peptone agar plates and dispensed on washed agar over a range of population densities. It is seen that at densities below 80 cells per mm.^{2} no centers appeared after 24 hours' incubation period. At high densities center formation was suppressed to some extent and at the optimal density, around 200 cells per mm.^{2}, a maximal number of centers was formed. The distribution of initiator cells to responder cells thus shown is 1:2100, in very close agreement with previous evaluations (Sussman and Noel, 1952). To determine the aggregative performances of cells in different growth phases, cultures grown on restricted medium were harvested at stages shown by the arrows in Figure 1. These stages were: 1 and 2, newly germinated myxamoebae at the end of the lag phase and/or at the beginning of the log phase; 3, at mid-log phase; 4, at the end of the log phase. The cells were washed by repeated centrifugation, suspended in salt solution, and replicate samples of 10,000 cells were delivered to washed agar over a range of population densities. It should be noted that even after thorough washing, the cells were contaminated to a slight extent with occluded bacteria. To avoid growth of the bacteria and therefore of the amoeba on the aggregation plates, streptomycin sulphate (Nutritional Biochemical) was added to the agar in final concentration of 0.5 gm. per liter. Unpublished experiments have shown that streptomycin prevents growth of the myxamoebae under these conditions but does not interfere in any observable manner with aggregation or fruit construction.

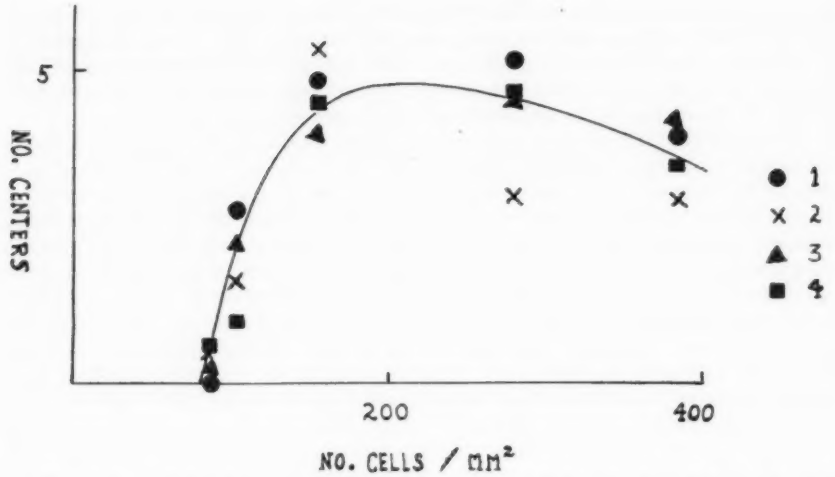

FIGURE 2. Relation between center formation and population density. Cell samples 1–4 were taken at times indicated by the arrows in Figure 1 and were washed by centrifugation and suspended in salt solution. Aliquots containing 10,000 cells were dispensed on washed agar over a range of densities. The densities were checked by direct observation with an ocular grid. The number of aggregative centers were counted after 24 hours. The ordinate shows the number of centers per aliquot.

A single experiment was performed at each of the stages mentioned. Figure 2 summarizes the data, each point representing the mean of at least six replicate samples. Aliquots containing relatively small numbers of cells (10,000) had to be used because of the practical limits imposed by the growth stages at which the cells were harvested. This magnified the random sampling error. Nevertheless, the points bracket the normal curve quite closely, and it is clear that no significant difference is apparent between the aggregative performance of cells in lag and log phases and that of cells from the stationary phase.

DISCUSSION

Evidence gained from a number of experimental directions has made it necessary to categorize aggregating myxamoebae as being either initiator cells that provide the aggregative stimulus or responder cells that answer it. One can account for this phenotypic divergence in at least two ways: a) The initiator cells arise spontaneously in the population via a stable genetic alteration. The ratio of initiator to responder cells is governed by selective pressures of the environment during the growth of the population. b) The matrix of conditions attending the cessation of growth induces a small proportion of the population to attain initiative capacity above the threshold necessary to attract responder cells under the conditions employed. This involves no prior genetic alteration but merely results from the exploitation of random physiological differences among the cells at that time. Thus, prior to the stoppage of growth, there are no initiator and responder cells *per se*.

The experiments described here support the second of these alternatives. In the mature fruiting body the spores represent a non-random sample of the original myxamoeboid population. This follows from the fact that the fates of the myxamoebae (whether they are to become spores, stalk cells, or basal disk cells) are determined by the order in which they entered the aggregates. The prestalk cells enter first, prespore cells next, and prebasal disk cells last (Bonner, 1944). Were the difference between initiator and responder cell due to a persistent, spontaneous, genetic change, one would expect that a newly germinated spore population should display a skewed distribution of initiator cells and that, during the subsequent growth of these cells, selective conditions would systematically alter the distribution until the customary ratio of 1:2200 was regained. In contrast, the second alternative does not carry with it the necessity of presuming these fluctuations in the initiator: responder ratio during the growth cycle. If the passage from a stage of active growth and synthesis to the stationary state itself evoked the random physiological variation that in turn determined whether a cell was to be an initiator or responder, then the distribution of initiators might be the same no matter what the past history of the cells.

The view is in accord with previously published results (Sussman, 1952, 1955) that indicate a spectrum of initiative capacity in the population. Thus in any system, the number of centers actually formed would depend both upon this spectrum and upon the level of sensitivity of the responder cells.

Summary

1. The growth kinetics of *Dictyostelium discoideum* have been determined on complete and restricted media.

2. Myxamoebae were taken from different parts of the lag and log phases and allowed to aggregate under conditions permitting quantitative examination. No difference could be discerned between the aggregative performance of these cells and that of stationary phase myxamoebae with respect to the number of centers formed or the population density response.

3. The bearing of these results upon the mechanisms of cellular differentiation during slime mold development is discussed.

LITERATURE CITED

Bonner, J. T., 1944. A descriptive study of the development of the slime mold, *D. discoideum*. *Amer. J. Bot.*, **31**: 175–182.

Bonner, J. T., 1947. Evidence for the formation of cell aggregates by chemotaxis in the development of the slime mold, *D. discoideum*. *J. Exp. Zool.*, **106**: 1–26.

Raper, K. B., 1940. Pseudoplasmodium formation and organization in *D. discoideum*. *J. Elisha Mitchell Sci. Soc.*, **56**: 241–282.

Sussman, M., 1951. Origin of cellular heterogeneity in the development of the cellular slime molds. *J. Exp. Zool.*, **118**: 407–418.

Sussman, M., and E. Noel, 1952. An analysis of the aggregation stage in the development of the slime molds, Dictyosteliaceae. I. The populational distribution of the capacity to initiate aggregation. *Biol. Bull.*, **103**: 259–262.

Sussman, M., 1952. An analysis of the aggregation stage in the development of the slime molds, Dictyosteliaceae. II. Aggregative center formation by mixtures of *D. discoideum* wild type and aggregateless variants. *Biol. Bull.*, **103**: 446–457.

Sussman, M., 1955. Fruity and other mutants of the cellular slime molds: a study of developmental aberrations. *J. Gen. Microbiol.*, **13**: 307–320.

STUDIES ON THE EFFECTS OF IRRADIATION OF CELLULAR PARTICULATES.[1] I. INHIBITION OF CYTOCHROME OXIDASE BY ULTRAVIOLET

HENRY T. YOST, JR., HOPE H. ROBSON AND IRWIN M. SPIEGLEMAN

Department of Biology, Amherst College, Amherst, Massachusetts

The effect of radiations of various types on enzyme systems has been the subject of numerous investigations. The exemplary work of Dale (1940, 1942), and of Barron and his associates (1949) has indicated that many enzymes are readily inactivated by ionizing radiation when sufficiently pure and when in dilute solution. Furthermore, their work clearly demonstrated that molecules, other than enzyme, when present in the irradiated solution, would protect the enzyme from inactivation. The current work on radiation "protection" has its origin in these findings. Such investigations have led to a clear picture of the action of ionizing radiations in the inhibition of various enzyme systems and have directed many investigators to seek the cause of gross radiation damage to organisms at the enzyme level. Similarly, much has been done to elucidate the nature of ultraviolet radiation damage to enzymes (McLaren, 1949; Lea, 1947). As early as 1897, Green had reported the destruction of diastase by ultraviolet. Nevertheless, less attention has been paid to the role of enzyme inactivation in lethal changes induced in cells by ultraviolet radiation. This is largely the result of the close correspondence of the nucleic acid absorption spectrum and most action spectra in the ultraviolet.

Although the disparity between the studies of "genetic" damage and enzyme damage is explicable in terms of the action spectra, it is surprising that in spite of the vast amount of work on the effects of radiations, little has been reported concerning their effects upon the particulate systems of cells. There are only two reports (both negative) of the effects of x-radiation on the cytochrome oxidase activity of particulates (Barron *et al.*, 1949; LeMay, 1951), and one report of the loss of the cytochromes from yeast after ultraviolet radiation (Raut, 1954). When one considers the vital role which has been assigned to particulates in the cellular metabolism, it would seem that any damage to this system would result in rather drastic changes in the cell. The remarkable similarity of the enzyme content of the particulates and the enzymatic mechanisms involving aerobic metabolism in widely differing organisms suggests a role in the continuity of the cell similar to that played by the chromosomes. For these reasons, it seemed advisable to make a study of the effects of various types of radiations on the mitochondrial fraction of the cellular particulates. The effects of "germicidal" ultraviolet radiation are reported in this paper.

For a test agent, cytochrome oxidase was chosen. This was done for three reasons: a depression in oxygen uptake has been observed by many workers following irradiation, cytochrome oxidase is closely bound to the particulate and there-

[1] This work was supported by a grant, No. C-2154, from the National Institutes of Health.

fore clearly forms part of its structure, and the recent reports of the enhanced effect of x-radiation after the addition of CO and CN (King, Schneiderman and Sax, 1952) indicate a possible role of cytochrome oxidase in radiation damage. Furthermore, cytochrome oxidase has been shown to be the terminal oxidase of many different organisms, and it seems evident that damage to this enzyme would result in alterations in the cellular metabolism of a very basic nature.

Materials and Methods

White laboratory rats were starved overnight and sacrificed by a blow on the head. The liver was removed and placed in cold 0.85% KCl, where a good deal of the blood was washed free. The liver was weighed and pressed through a bronze

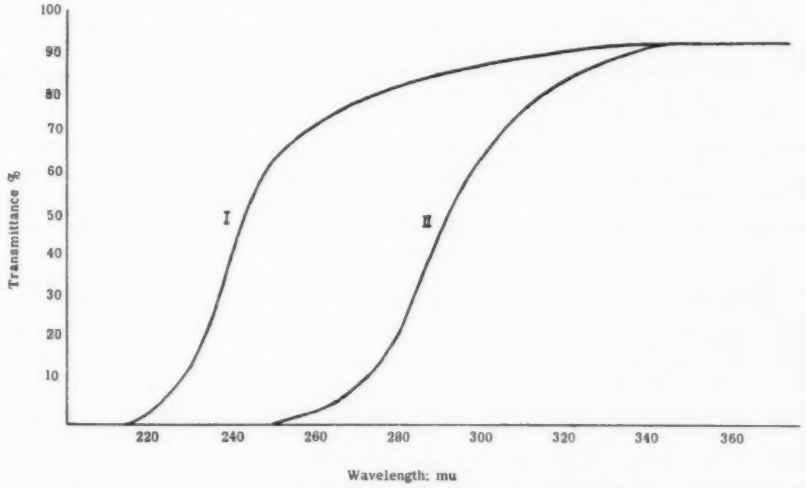

Figure 1. The transmission of the two filters used in this study. Filter I is Corning No. 7910 and Filter II is Corning No. 9700. Curves drawn from data supplied by the Corning Glass Co. and spot checked with a Beckman spectrophotometer.

sieve to remove connective tissue. The resulting mash was then suspended in cold 8.5% sucrose (50 ml.) and homogenized in a glass homogenizer with a "Teflon" pestle driven by a cone-drive stirring motor. The mitochondria were then separated from the rest of the homogenate by the method of differential centrifugation (Schneider, 1948). The mitochondrial fraction alone was kept. This fraction was washed twice with cold 8.5% sucrose, and the final sediment was suspended in 2.5 ml. sucrose solution per gram of liver. All centrifugations were carried out in a refrigerated centrifuge at 4° C.

For irradiation, a sample of the suspension was diluted 1 in 5, 1 in 10, or 1 in 15 with the same sucrose solution. (The dilutions used in each case are given with the data.) Two and five-tenths ml. were placed in a quartz flask rotated in the ultraviolet beam by a slow speed stirring motor; 2.5 ml. were kept in a Pyrex test

tube, in the cold, as a control. During the period of irradiation, the quartz flask was partially submerged in an ice-water bath maintaining a temperature under 5° C. The flask was kept 11.5 or 18.5 cm. from a G.E. germicidal lamp for all radiations. The 18.5-cm. position is referred to as the low intensity position in the rest of the paper. The intensity of the radiation was 5500 ergs/cm.^{2}/sec. at the 11.5-cm. position as measured by a G.E. light meter with ultraviolet cell. In the filter studies, different filters were placed between the source and the flask. The transmission of the two filters used is given in Figure 1. Filter I is a Corning ultraviolet transmitting filter (No. 7910) which transmits principally those wave-lengths above 2400 Å; and Filter II (Corning No. 9700) transmits principally those wave-lengths above 2800 Å. In a few experiments, the mitochondrial suspension was irradiated in an open petri plate to test the effect of the rotation in the quartz flask. When this was done, controls were also kept in the cold in an open petri plate.

In the studies done with whole cells, cells of *Parthenocissus* crown gall grown in tissue culture were used. These cells were spread out on moist filter paper in a petri dish and exposed to the ultraviolet radiation as above. The cells were then collected and the mitochondrial fraction obtained in the same manner as that used for rat liver, with the exceptions that the tissue was ground with sand in a mortar and the final suspension was made up in a total of 2 ml. sucrose. The small fraction obtained in this way showed cytochrome oxidase activity. In cases where the oxygen uptake of whole cells is reported, the results represent the endogenous respiration. All radiations of whole cells were carried out in the same way and at the same distance (11.5 cm.) from the source.

The cytochrome oxidase activity was estimated manometrically by the method of Hogeboom, Claude and Hotchkiss (1946). The main compartment of each vessel contained: 0.35 ml. of the mitochondrial suspension, 0.1 ml. Sorenson phosphate buffer (pH 7.4), 1 ml. 1.3×10^{-4} *M* cytochrome-*c* (Sigma, horse-heart) in 0.85 % NaCl, and 0.15 ml. 0.005 *M* $AlCl_3$. The center well contained 0.2 ml. 5 N KOH; and the side arm held the reducing agent, 0.15 ml. 0.228 *M* sodium ascorbate. In studies concerning the effects of added nucleotides, each flask contained 5 μM AMP (adenosine-5-phosphoric acid, free acid—Sigma), ADP (adenosine diphosphate, sodium salt—Sigma), or ATP (adenosine triphosphate, neutral sodium salt —Schwartz). In these cases, as in the case of the determination of the effect of pH on the enzyme, the buffer in the flask was changed. In the experiments with the nucleotides the buffer was increased in an attempt to keep the final pH of the reaction at 7.4. In the pH series, various phosphate buffers were used to give the desired range. In the determination of the oxygen uptake of the whole cells the vessels contained only cells and one ml. of water. The center well contained alkali as above. All determinations were made at 38° C. with the exception of the whole cells and the ones made with ATP. These two latter were done at 30° C.

Attempts to photoreactivate the system were made by placing the irradiated suspension under two 250-watt fluorescent lights for various lengths of time, as indicated in the tables. In some cases the exposure to light was made in an ice bath and in others the experiments were carried out at room temperature. The suspension was exposed to the light in a petri plate with the cover left on, and with two thicknesses of window glass between the petri plate and the source. This was done to remove any ultraviolet radiation from the fluorescent tubes.

All assays were made in triplicate. In addition, most experiments were re-

peated three or more times; some of the less important points on the dosage curve were repeated only twice.

Results

The data presented in Table I show the effectiveness of ultraviolet radiation in the inactivation of cytochrome oxidase. In this case, representative experiments are shown (chosen to correspond to the average value for several runs). It can be seen that 20 minutes of radiation are sufficient to reduce the oxidase activity to about 40% of the controls, and that at one hour only 20% of the activity is left. These values were obtained using Filter I; without the filter the source is only

Table I

The effect of ultraviolet radiation and of rotation upon cytochrome oxidase activity

(Preparation diluted 1 : 5)

Treatment	μL O_2	Per cent inactivation
Control	365.4	
20 min. UV	163.6	55.2%
Control	159.4	
60 min. UV	29.9	81.2%
Control	134.7	
60 min. rotation	124.0	7.9%

slightly more effective, the difference being due to the absorption of the filter. Table I also presents data on the effect of rotating the suspension in the quartz flask without radiation. This was done to see if there was an inactivation of the oxidase by this procedure. This particular experiment was repeated ten times, and there is an average inactivation of 8.2% with one hour rotation. This is very slight when compared to the effect of the radiation and can be ignored for most of the studies (wherever Filter I is used).

Table II presents the data obtained when different filters were used. The activity is expressed as per cent of the controls and represents an average value of all experiments. It is evident that Filter I (wave-lengths above 2400 Å) is much more effective than Filter II (wave-lengths 2800 Å). One hour of radiation with Filter

Table II

Relative effectiveness of filtered ultraviolet radiation

(Preparation diluted 1 : 5)

Filter*	Treatment	Per cent control activity
I	4 min.	80.5%
I	10 min.	63.8%
I	60 min.	19.0%
II	60 min.	81.8%
II	180 min.	62.5%

* Filter I transmits mostly wave-lengths above 2400 Å; Filter II transmits mostly above 2800 Å (see Fig. 1).

II produced about as much inactivation as 4 minutes with Filter I; three hours with Filter II is equivalent to 10 minutes with Filter I. It seems that Filter II is only about $\frac{1}{15}$ as effective as Filter I (about 6%).

Figure 2 presents the relation between the inactivation of cytochrome oxidase and the dosage of ultraviolet. The curve was obtained using Filter I. This curve is a composite and should be considered only as a rough indication of the course of inactivation. All the points represent average values from several experiments. There is some variability expected in this case, since if one uses the same rat liver for all points on the curve, the controls are slowly losing activity with age, and if one uses different preparations for the different points, there is variation in the cytochrome oxidase concentration of the initial suspensions. However, it is clear that the initial inactivation of the enzyme is rapid, but the slope of the curve becomes more gradual after 20 minutes, reaching a maximum inactivation at about

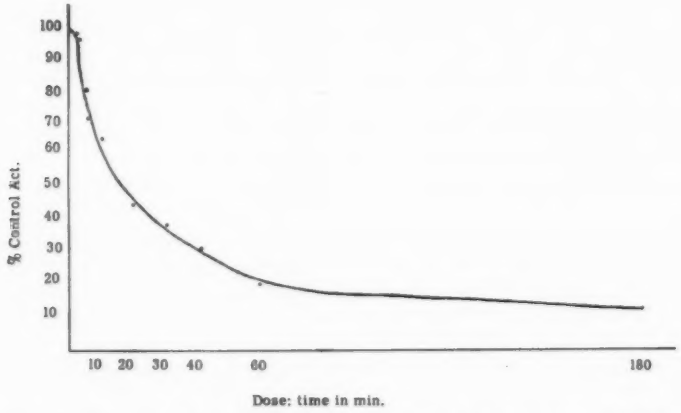

FIGURE 2. Inactivation of cytochrome oxidase by ultraviolet radiation. Radiation at high intensity; filter I; preparation diluted 1 in 5. Activity is expressed as per cent of the control activity.

one hour. This curve is similar to the inactivation curves obtained with microorganisms, and the shape is probably due to the gradual increase in protection against radiation by the inactivated enzyme. At the high intensity of radiation used in these experiments, it was not possible to determine the threshold value for the inactivation. Therefore the intensity was reduced by moving the flask away from the source. The data obtained with this lower intensity radiation are presented in Figure 3. It is evident that there is a definite threshold value for the inactivation, and that this value varies with the age of the preparation. There is distinct evidence for a slight increase in the activity of older preparations when given very low doses of ultraviolet. With higher doses (as in Figure 2) there is never any indication of an increase in activity.

Table III presents data obtained in attempts to reactivate the irradiated cytochrome oxidase. It is evident that there is neither photoreactivation nor reactiva-

tion with ADP or ATP. During the attempts to reactivate the preparation with ADP and ATP, a slight stimulation of both the controls and the irradiated preparation by the ADP and a slight inhibition by ATP was noted. It was thought that this might be an indication of the participation of these compounds in the oxidation of cytochrome-*c* as suggested recently by the work of Lehninger, Hassan and Sudduth (1954). However, it is extremely difficult to maintain a constant pH in the vessels during a test made with these compounds, and subsequent investigation indicated that the enzyme is extremely sensitive to slight variations in pH. The relationship between cytochrome oxidase activity and pH is given in Table IV. There seems to be no effect of the added ADP or ATP which is not the result of

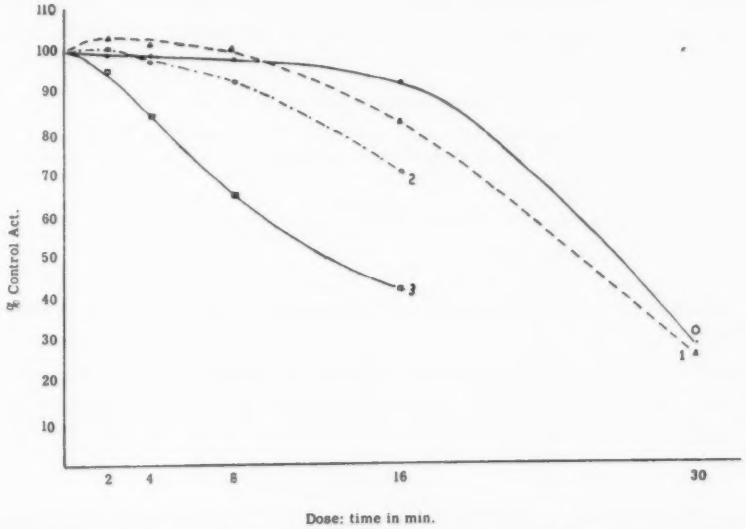

FIGURE 3. Inactivation of cytochrome oxidase preparations of varying ages by ultraviolet radiation. Source at lower intensity position; filter 1; preparation diluted 1 in 15. ·——· preparation radiated same day extracted; △– – –△ preparation radiated one day later; ○—·—○ preparation radiated two days later; □——□ preparation radiated three days later.

a shift in the pH. These data seem to indicate that it is not the adenylic acid system which is being inactivated by the ultraviolet. The absence of photoreactivation in such a simple system is not surprising.

To determine the effect of ultraviolet radiation on the cytochrome oxidase activity of irradiated whole cells, cells of *Parthenocissus* crown gall were irradiated and the particulate fraction isolated after radiation. The data from these experiments are summarized in Table V. It can be seen that although 5 minutes of radiation reduces the endogenous oxygen uptake of the intact cells 17%, it takes one to two hours to achieve equivalent inactivation of the particulates removed from the irradiated cells. It would seem that the cytochrome oxidase of these cells is not greatly affected by ultraviolet radiation so long as the radiation is carried out on

TABLE III

Failure to reactivate irradiated cytochrome oxidase

(Preparation diluted 1 : 5)

Treatment	% control activity
10 min. UV	65.6%
10 min. UV + ADP	67.2%
10 min. UV	66.3%
10 min. UV + ATP	61.4%
20 min. UV	44.8%
20 min. UV + ADP	46.3%
20 min. UV	44.6%
20 min. UV + ATP	39.8%
(Preparation diluted 1 : 10)	
40 min. UV	36.7%
40 min. UV + 20 min. light*	33.2%
2 min. UV	74.4%
2 min. UV + 20 min. light†	70.0%
20 min. light†	94.2%

* Continuous light; treatment at 5° C.

† Two 10-minute light periods separated by 10 minutes of dark; treatment at room temperature.

the intact cells. Control experiments indicate no difference in the sensitivity of particulates radiated after being extracted from the cells. It is apparent that the cytochrome oxidase is protected by the rest of the cell from the radiation damage.

DISCUSSION

The data presented above indicate that cytochrome oxidase in the isolated cellular particulate is very sensitive to ultraviolet radiation. Furthermore, these data indicate that ultraviolet wave-lengths shorter than 2800 Å produce this effect. Reference to Table II indicates that Filter II is only about 6% as effective as Filter I. At these low values of inactivation, the effect of rotation of the quartz flask becomes appreciable. Table I shows that an hour of rotation decreases that activity of the preparation about 8%. Therefore, one may assume that the value of rela-

TABLE IV

The effect of pH on the activity of cytochrome oxidase

(Preparation diluted 1 : 10)

pH	Oxygen uptake (μL O_2)	% activity of 7.4
7.4	195.6	100.0
7.3	177.0	90.5
7.2	141.8	72.5
7.1	127.4	65.1
7.0	115.0	58.8

tive effectiveness given for Filter II is on the high side. From a comparison of the transmission of the two filters (Figure 1), and from a comparison of their relative effectiveness, it would seem that the effect is induced by wave-lengths in the region 2600–2700 Å. However, the source used in these studies was a germicidal lamp, and therefore most of the emitted energy was in the region of 2537 Å. One would expect a protein to be denatured by all wave-lengths below 3100 Å, with maximum effects at the absorption maxima of the protein. To test the efficiency of the source and filters on the inactivation of a protein, crystalline urease prepared by the method of Sumner (1926) and Dounce (1941) was irradiated in the same manner as the cytochrome oxidase. It was found that with Filter I, 10 minutes of radiation were necessary to produce 50% inhibition of the enzyme; to achieve similar inactivation required 60 minutes of radiation with Filter II. Therefore, it is evident that for the inactivation of a more simple protein molecule, urease, the filters have an efficiency ratio of 6. Since urease has been reported to have an absorption

TABLE V

The effect of irradiation of whole cells upon the oxygen uptake of the cells and of isolated particulates

Test	Treatment	Oxygen uptake (μL O_2)	% inactivation
Cells	Control	48.4	
	5 min. UV	40.4	17%
Cells	Control	48.3	
	120 min. UV	20.8	57%
Particulates	Control	48.1	
	60 min. UV	43.9	9%
Particulates	Control	56.6	
	60 min. UV	47.1	17%
Particulates	Control	49.2	
	120 min. UV	43.7	11%

maximum typical of simple proteins in the region of 2800 Å, and since the action spectrum for destruction of the enzyme and the absorption spectrum coincide (Kubowitz and Haas, 1933), this efficiency ratio provides us with a measure of the radiation from this source in the region 2800 Å. Since the destruction ratio for cytochrome oxidase is 15:1 with these same filters, it must be concluded that the wave-lengths below 2800 Å are about two times as efficient as those above. Therefore it seems possible to conclude that the cytochrome oxidase activity of isolated particulates is readily inactivated by wave-lengths in the region of 2600–2700 Å. Since some effect is obtained with Filter II, the wave-length cannot be lower than 2600 Å.

The fact that wave-lengths in the vicinity of the so-called "nucleic acid peak" are capable of deactivating cytochrome oxidase suggested that some nucleotide associated with the activity of the oxidase was being destroyed. Recent work by Lehninger, Hassan and Sudduth (1954) has demonstrated the formation of ATP during the oxidation of cytochrome-*c*. It was thought that perhaps either ADP or ATP was destroyed by the radiation and that this destruction was acting to inhibit

the over-all reaction. Attempts were made to reactivate the preparation by the addition of both of these compounds, without success. It is possible that there is a nucleotide bound to the enzyme, however, which is destroyed and cannot be replaced by external sources; unfortunately such an hypothesis is difficult to test. As pointed out above, in the course of these investigations it was discovered that the pH had a very great effect upon the enzyme activity, more than could be accounted for by changes in the cytochrome-*c*. It is possible that the ultraviolet acts upon the particulate in such manner that the internal pH is shifted. However, this seems unlikely when the effect remains after prolonged exposure to buffer at the optimal pH.

It might also be suggested that the ultraviolet is destroying the structure of the particulate and thus inactivating the enzyme. This seems unlikely in view of the fact that the enzyme can be solubilized and retain its activity (Eichel *et al.*, 1950). Experiments done with the solubilized preparation indicate that the oxidase is inactivated by ultraviolet radiation in the same manner as in the intact particle. This also seems to eliminate the possibility that the effect is upon some other enzyme in the oxidative pathway, whose loss inhibits cytochrome oxidase. The best assumption, at present, seems to be that the effect is upon the oxidase molecule directly. When the data presented in Figure 3 are considered, it becomes evident that at low doses there is a stimulation of oxygen uptake, under certain conditions. This effect is observed most readily in a preparation that has aged for one or two days in the cold. Most frequently this effect is associated with an increase in the activity of the controls, when compared to those of the preceding day. The simplest explanation of this "aging" effect is that the permeability of the particulate membrane is increasing with time. The effect of the ultraviolet radiation may be to hasten (or increase) this change. It should be noted that this effect is not limited to old preparations. Sometimes it is observed with fresh preparations, generally with very low doses. Apparently this effect depends upon the conditions which prevail in the cell before extraction. It will be noted that the increase with low doses of ultraviolet disappears as the preparation gets older. At this time the control activity is also dropping, indicating a loss of activity by the cytochrome oxidase, for which further shifts in permeability would not compensate. These observations may provide an explanation for the reports of stimulation of oxygen uptake by ultraviolet radiation in whole cells. In the whole cell, as can be seen in Table V, the oxidase molecule is protected by other cellular constituents. This represents an effective lowering of the dose of ultraviolet, and may, under some conditions, result in a stimulation of the activity of cytochrome oxidase. It is evident that the effect of the ultraviolet in increasing the activity of the cytochrome oxidase may not be an effect on the permeability of the particulate membrane; however, since the effect becomes more pronounced with age, it seems to be an effect on something other than the oxidase molecule itself, permeability being the most likely suspect.

It is evident that further analysis depends upon a determination of the absorption spectrum of the cytochrome oxidase molecule and an elucidation of the action spectrum for its inactivation. Neither of these problems is simple. Measurements of the absorption spectrum of a suspension of isolated particulates show no clear peaks in the ultraviolet below 2800 Å. There is a general peak between 2800–2900 Å, corresponding to the protein maxima, but below this the absorption tapers off gradually. The difference in the absorption between 2800 and 2600 Å is rather small, indicating general absorption in the ultraviolet. Attempts were made to con-

centrate the cytochrome oxidase in its solubilized form by the method of Wainio *et al.*, (1948). Isolation of the enzyme resulted in a clear (yellow) preparation having reasonable activity. However, the preparation is completely opaque to wavelengths below 2700 Å, largely the result of the extractant, desoxycholic acid. Removal of the extractant results in the precipitation of the enzyme in an insoluble form. Further work in this direction awaits a new method for the purification of cytochrome oxidase. The only indication that the molecule has an absorption peak at 2600–2700 Å is the CO spectrum obtained by Warburg and Negelein (1929). They obtained a strong peak in this region. Measurements of the action spectrum for the inactivation of the enzyme are complicated by the fact that the intensity of most monochrometers is too low to give meaningful results. The radiation time becomes excessive, and the preparation is destroyed by other factors than the radiation. A sufficiently intense monochrometer is not available in this laboratory at the present time.

The fact that ultraviolet radiation in the region 2600–2700 Å inhibits cytochrome oxidase activity suggests that one of the causes of cell death may be the destruction of this enzyme. It is well known that ultraviolet radiation in relatively low doses blocks the cell division mechanism, and at higher doses causes the death of cells. This effect has been attributed to a change in the nucleic acids of the cells. It seems quite possible that the effect is on the enzyme systems of the cells. Inactivation of one of the major terminal oxidases would undoubtedly disturb the metabolism of the cell and possibly result in death. Attempts were made to test this idea using cells of *Parthenocissus* crown gall grown in tissue culture. These cells were chosen because they can be grown in large quantities, and they lack the pigments which would interfere with the absorption of the ultraviolet. Attempts were made to study this problem with rat liver cells, but even with repeated washing of the cells they could not be totally freed of blood. The plant cells seemed to be ideal. The cell division mechanism is inhibited with relatively low doses of ultraviolet (two minutes), and the respiration is also inhibited. However, as can be seen in Table V, these effects cannot be attributed to an effect upon the cytochrome oxidase. It would appear that the effect of the ultraviolet is upon some step preceding the terminal oxidase or upon some other enzyme acting as a terminal oxidase in these cells. As cytochrome oxidase may not be the major terminal oxidase, the test is inconclusive. Tissue which is normally exposed to ultraviolet radiation and which is sufficiently clear to permit penetration (skin, cornea, etc.) is difficult to come by in sufficient quantities to extract the particulates. At the present time no final conclusion can be reached on this point. It does seem probable that exposed tissues lose the activity of cytochrome oxidase in cells in which this is a major oxidase. The consequence of this loss may be drastic or slight depending upon the degree of destruction of the enzyme and the other mechanisms present in the cell to carry on the oxidative metabolism.

Summary

Data are presented which indicate that ultraviolet radiation of 2600–2700 Å is effective in the inactivation of cytochrome oxidase in intact cellular particulates. The effect seems to be upon the oxidase molecule itself. It is suggested that this could be one cause of cell death from ultraviolet radiation.

LITERATURE CITED

BARRON, E. S. G., S. DICKMAN, J. A. MUNTZ AND T. P. SINGER, 1949. Studies on the mechanism of action of ionizing radiations. I. Inhibition of enzymes by X-rays. *J. Gen. Physiol.*, **32**: 537–552.

DALE, W. M., 1940. The effect of X-rays on enzymes. *Biochem. J.*, **34**: 1367–1373.

DALE, W. M., 1942. The effect of X-rays on the conjugated protein d-amino acid oxidase. *Biochem. J.*, **36**: 80–85.

DOUNCE, A. L., 1941. An improved method for recrystallizing urease. *J. Biol. Chem.*, **140**: 307–308.

EICHEL, B., W. WAINIO, P. PERSON AND S. COOPERSTEIN, 1950. A partial separation and characterization of cytochrome oxidase and cytochrome-*b*. *J. Biol. Chem.*, **183**: 89–103.

GREEN, R., 1897. On the action of light on diastase and its biological significance. *Proc. Royal Soc. London, Ser. B*, **61**: 25–28.

HOGEBOOM, G., A. CLAUDE AND R. HOTCHKISS, 1946. The distribution of cytochrome oxidase and succinoxidase in the cytoplasm of the mammalian liver cell. *J. Biol. Chem.*, **165**: 615–629.

KING, E. D., H. A. SCHNEIDERMAN AND K. SAX, 1952. The effects of carbon monoxide and oxygen on the frequency of X-ray induced chromosome aberrations in *Tradescantia*. *Proc. Nat. Acad. Sci.*, **38**: 34–43.

KUBOWITZ, F., AND E. HAAS, 1933. Über das Zerstörungsspektrum der Urease. *Biochem. Zeitschr.*, **257**: 337–343.

LEA, D. E., 1947. Actions of radiations on living cells. The Macmillan Co., New York.

LEHNINGER, A. L., M. U. HASSAN AND H. C. SUDDUTH, 1954. Phosphorylation coupled to the oxidation of ascorbic acid by isolated mitochondria. *J. Biol. Chem.*, **210**: 911–922.

LEMAY, 1951. Effect of X-radiation on succinoxidase of rat kidney. *Proc. Soc. Exper. Biol. Med.*, **77**: 337–339.

MCLAREN, A. D., 1949. Photochemistry of enzymes, proteins and viruses. *Adv. in Enzymol.*, **9**: 75–170.

RAUT, C., 1954. Heritable nongenic changes induced in yeast by ultraviolet light. *J. Cell. Comp. Physiol.*, **44**: 463–475.

SCHNEIDER, W., 1948. Intracellular distribution of enzymes. III. The oxidation of octanoic acid by rat liver fractions. *J. Biol. Chem.*, **176**: 259–266.

SUMNER, J. B., 1926. The isolation and crystallization of the enzyme urease. *J. Biol. Chem.*, **69**: 435–441.

WAINIO, W., S. COOPERSTEIN, J. KOLLEN AND B. EICHEL, 1948. The preparation of soluble cytochrome oxidase. *J. Biol. Chem.*, **173**: 145–152.

WARBURG, O., AND R. NEGELEIN, 1929. Über das Absorptionsspektrum des Atmungsferments. *Biochem. Zeitschr.*, **214**: 64–100.

BIOLOGY MATERIALS

The Supply Department of the Marine Biological Laboratory has a complete stock of excellent plain preserved and injected materials, and would be pleased to quote prices on school needs.

PRESERVED SPECIMENS

for

Zoology, Botany, Embryology, and Comparative Anatomy

LIVING SPECIMENS

for

Zoology and Botany

including Protozoan and Drosophila Cultures, and Animals for Experimental and Laboratory Use.

MICROSCOPE SLIDES

for

Zoology, Botany, Embryology, Histology, Bacteriology, and Parasitology.

CATALOGUES SENT ON REQUEST

Supply Department

MARINE BIOLOGICAL LABORATORY

Woods Hole, Massachusetts

CONTENTS

Page

BERG, WILLIAM E.

Cytochrome oxidase in anterior and posterior blastomeres of Ciona intestinalis 1

BURDEN, CHARLES E.

The failure of hypophysectomized Fundulus heteroclitus to survive in fresh water 8

DAN, KATSUMA, AND KAYO OKAZAKI

Cyto-embryological studies of sea urchins. III. Role of the secondary mesenchyme cells in the formation of the primitive gut in sea urchin larvae 29

DEHNEL, PAUL A.

Growth rates in latitudinally and vertically separated populations of Mytilus californianus 43

JOHNSON, MARTIN W., AND ROBERT J. MENZIES

The migratory habits of the marine gribble Limnoria tripunctata Menzies in San Diego habor, California 54

KRUGELIS-MACRAE, EDITH

The occurrence of porphyrin in the planarian 69

LUDWIG, DANIEL, AND MARY C. BARSA

Oxygen consumption of whole insects and insect homogenates 77

NUTTING, W. L.

Reciprocal protozoan transfaunations between the roach, Cryptocercus, and the termite, Zootermopsis 83

SUSSMAN, MAURICE

On the relation between growth and morphogenesis in the slime mold Dictyostelium discoideum 91

YOST, HENRY T., JR., HOPE H. ROBSON AND IRWIN M. SPIEGLEMAN

Studies on the effects of irradiation of cellular particulates. I. Inhibition of cytochrome oxidase by ultraviolet 96